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# Structural and Functional Characterization of Full-Length Heparin-Binding Growth Associated Molecule

**Brian S. Hampton, Daniel R. Marshak,* and Wilson H. Burgess**

American Red Cross, Laboratory of Molecular Biology, Jerome H. Holland Laboratory for the Biomedical Sciences, Rockville, Maryland 20855; and the *Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

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Heparin-binding growth-associated molecule (HB-GAM) was purified from adult bovine brain and chicken heart. The yield of HB-GAM is increased by 5- to 10-fold when 250 mM NaCl is added to the homogenization buffer, indicating that HB-GAM may exist as a complex with an insoluble component of the tissue. The complete amino acid sequence of the brain-derived HB-GAM was established by automated Edman degradation of the intact protein and chemically or enzymatically derived fragments. The mass of bovine HB-GAM as determined by plasma desorption time-of-flight mass spectrometry is 15 291 mass units, which compares favorably with the calculated mass of 15 289 based on the amino acid sequence. Therefore, HB-GAM has not undergone any major post-translational modifications other than cleavage of the signal peptide. These results indicate that previous amino acid sequence analysis of this protein was carried out using truncated HB-GAM. Full-length HB-GAM is not a mitogen for Balb/3T3 clone A31, Balb MK, NRK, or human umbilical vein endothelial cells. HB-GAM does, however, have adhesive properties and neurite extension activity for chick embryo cerebral cortical derived neurons when presented to these cells as a substrate. HB-GAM had little neurite extension activity when presented as a soluble factor.

## INTRODUCTION

Heparin-binding growth-associated molecule (HB-GAM)[1] (also referred to as ECGF-LP, p18, HBGF-8, HBNF, or pleotrophin) is a basic cysteine-rich heparin-binding protein that has been isolated from brain (Burgess *et al.*, 1985; Rauvala, 1989; Kuo *et al.*, 1990) and placenta (Milner *et al.*, 1989). The cDNA that encodes this protein has been cloned and sequenced from several species (Li *et al.*, 1990; Merenmies and Rauvala, 1990). This protein is expressed in a temporal- and spacial-specific manner in the developing embryo and is expressed intensely in the brain of postnatal rats (Rauvala, 1989). HB-GAM is related structurally to MK, a protein expressed by embryonal carcinoma cells after treatment with retinoic acid (Kadomatsu *et al.*, 1988) and to a protein isolated from chick embryo basement membranes (Vigny *et al.*, 1989; Urios *et al.*, 1991). Several features are common among these proteins. For example, they are all basic proteins rich in lysine residues and they have a high apparent affinity for heparin. The relative positions of 10 cysteine residues are conserved and based on the cDNA sequences; the open reading frames encode consensus signal peptide sequences that would allow these proteins to be secreted.

During the course of purification of bovine brain-derived acidic fibroblast growth factor (FGF-1),[2] originally referred to as endothelial cell growth factor, we identified a heparin-binding protein that was related immunologically and had an apparent molecular weight similar to FGF-1; hence, it was named endothelial cell growth factor-like protein (ECGF-LP) (Burgess *et al.*,

[1] Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; ECGF-LP, endothelial cell growth factor-like protein; FGF-1, acidic fibroblast growth factor; HB-GAM, heparin-binding growth-associated molecule; HPLC, high-pressure liquid chromatography; NTF, neurotrophic factor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RI-HB, retinoic acid-induced heparin binding; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane.

[2] The abbreviations used for fibroblast growth factors are based on the conclusions of the New York Academy of Science conference, La Jolla, California, 1991.

1985). Further analysis revealed that this protein had a high content of lysine residues, and amino-terminal sequence analysis indicated that this protein was not related structurally to members of the FGF family. Recently, the cDNA sequence of rat HB-GAM was reported (Merenmies and Rauvala, 1990) and the deduced amino acid sequence from this cDNA revealed that ECGF-LP was the bovine homolog of HB-GAM.

The cDNA sequence of HB-GAM contains an open reading frame that encodes for a putative signal peptide that would be cleaved during processing leaving a 136 amino acid polypeptide that may be secreted. To date, amino acid sequence analysis has failed to identify a 136 amino acid form of the protein (Kuo *et al.*, 1990; Bohlen *et al.*, 1991). Rather, the complete amino acid sequence of bovine HB-GAM was reported to consist of 119 amino acids (Kuo *et al.*, 1990), suggesting the possibility that proteolysis of the carboxyl-terminus had occurred. More recently, the documented amino acid sequence of a 114 amino acid form of HB-GAM was reported (Bohlen *et al.*, 1991). In that report, the amino acid composition analysis indicated that a 136 residue form of the protein may exist; however, amino acid sequence extending beyond residue 114 was not obtained.

The two biological activities reported for HB-GAM are a mitogen for mouse or rat fibroblasts and a neurite extension factor for rat brain neurons and a rat pheochromocytoma cell line (PC-12). However, there has not been a consensus in the literature with regard to the assignment of these activities. Ravaula (1989) determined that rat brain-derived HB-GAM was a neurite extension factor for rat neurons in vitro. Milner *et al.* (1989) described the isolation of HB-GAM from an acetone powder preparation of bovine placenta and reported it to be an acid labile mitogen for NIH 3T3 cells with a potency equivalent to that of FGF-1. However, sufficient evidence was not presented to exclude the possibility of contamination by other heparin-binding growth factors such as basic fibroblast growth factor. Indeed, ~50% of the mitogenic activity in their preparation did not correlate with the elution position of the major UV absorbing peak (from the final purification step) but could be found in the side fractions of this peak (Milner *et al.*, 1989). Subsequent experiments by Li *et al.* (1990) showed that when the cloned gene is transiently expressed in COS cells, the cell extract has relatively little mitogenic activity on the basis of the level of expression of recombinant HB-GAM. Other studies that reported mitogenic activity of bovine HB-GAM (Bohlen *et al.*, 1988; Bohlen and Gautschi, 1989) have recently been retracted (Bohlen *et al.*, 1991). We reported previously (Burgess *et al.*, 1985) that a protein now known to be identical to bovine brain-derived HB-GAM was not a mitogen for mouse lung capillary endothelial cells. Similarly, it was reported by Kuo *et al.* (1990) that their preparation of HB-GAM was not a mitogen for NIH 3T3 cells but is a neurite extension factor for PC-12 cells. However, it is possible that the lack of mitogenic activity in these preparations is related to carboxyl-terminal truncations (Kuo *et al.*, 1990; Bohlen *et al.*, 1991) or to acidic conditions used during purification (Burgess *et al.*, 1985). To resolve these questions we extended our studies on the isolation and characterization of this protein.

In this report we describe the complete primary structure of bovine and avian HB-GAM. On the basis of the primary structure, we show that these mature proteins consist of 136 or 135 amino acids, respectively, and present evidence that the truncated forms of HB-GAM isolated previously (Kuo *et al.*, 1990; Bohlen *et al.*, 1991) are an artifact of purification rather than post-translational modification. We also demonstrate its neurotrophic activity on chick embryo cerebral cortical neurons and its lack of mitogenic activity on BALB/3T3, BALB/MK, NRK, and human umbilical vein endothelial cells as compared with FGF-1. We show that HB-GAM is present in adult tissues of non-neuronal origin and present direct evidence that HB-GAM has not undergone any significant post-translational modification other than cleavage of the amino-terminal signal peptide. In contrast to previous reports (Burgess *et al.*, 1985; Kuo *et al.*, 1990; Bohlen *et al.*, 1991), the lack of mitogenic activity of our preparations of HB-GAM cannot be attributed to acidic treatment or carboxyl-terminal truncations of the protein.

## MATERIALS AND METHODS

### *Purification*

HB-GAM was purified essentially as described (Burgess *et al.*, 1985) with some modifications. Six adult bovine brains were homogenized in 1.3 volumes (wt/vol) of 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.5, 50 mM EDTA, 250 mM NaCl. The homogenate was clarified of insoluble material by centrifugation at 13 500 × *g* for 60 min. Ammonium sulfate was added to the supernatant to 50% saturation, stirred for 60 min, centrifuged at 13 500 × *g* for 60 min, and the pellet was discarded. Additional ammonium sulfate was added to 95% saturation, stirred for 60 min, and centrifuged at 13 500 × *g* for 60 min. The resulting pellet was resuspended in 10 volumes of H/S buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA) and stirred with 40 ml of heparin-Sepharose (Pharmacia, Piscataway, NJ) equilibrated with H/S buffer. After 60 min the heparin-Sepharose was washed on a scintered glass funnel with 1000 ml of H/S buffer and then with 1000 ml of H/S buffer containing 500 mM NaCl. The resin was transferred to a 1.5-cm diameter column and bound proteins were eluted with a gradient from 0.5 to 1.5 M NaCl and 10-ml fractions were collected.

The fractions that eluted between 0.8 and 1.1 M NaCl were pooled, diluted 10-fold in 50 mM sodium phosphate buffer, pH 6.0, and fractionated further by ion exchange chromatography using a Waters 840 HPLC system (Waters, Medford, MA) and a 5 mm × 5 cm MONO-S column (Pharmacia) equilibrated in the same buffer. Proteins were eluted with a linear gradient from 0 to 1.0 M NaCl over 50 min. Final purification of HB-GAM was achieved by ion exchange chromatography on an Aquapore (Browlee Labs, Santa Clara, CA) CX-300 2.1 mm × 10 cm column equilibrated with 50 mM sodium phosphate pH 7.0 and eluted as described above. Purification of HB-GAM from chicken heart was done as previously described for the purification of FGF-1 from bovine heart (Sasaki *et al.*, 1989). Recombinant human

FGF-1 was used in the bioassays and was purified as described previously (Burgess *et al.*, 1990). Briefly, cultures of *Escherichia coli* bearing a plasmid that encodes residues 1–154 of human FGF-1 (kindly provided by Michael Jaye: Rhone-Poulenc Rorer, King of Prussia, PA) were grown and FGF-1 was purified by heparin-Sepharose affinity chromatography and reversed-phase chromatography. These preparations were judged to be >95% pure on the basis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

### *Determination of the Primary Structure*

Before enzymatic digestion, the cysteine residues of HB-GAM were modified. Briefly, reduction was carried out with 59 mM 2-mercaptoethanol in 6 M guanidine-HCl, 250 mM Tris-HCl, pH 8.5, 1 mM EDTA at 37 μC for 60 min. 4-Vinyl-pyridine was then added to a final concentration of 440 mM and incubated at room temperature for 90 min. Enzymatic digestions of 1–3 nmol of HB-GAM with endoproteinase Lys-C, endoproteinase Glu-C, or endoproteinase Asp-N were carried out according to the manufacturers instructions at a ratio of 1:10 enzyme to protein (wt/wt). The buffer used in the digestions with endoproteinase Glu-C was 25 mM ammonium bicarbonate (pH 7.8) to limit this enzyme's specificity to cleavage primarily at the carboxyl-terminal side of glutamic acid. Additionally, all digestion buffers contained 10% (vol/vol) acetonitrile. HB-GAM was also cleaved at methionine residues by cyanogen bromide. Peptides generated by enzymatic or chemical cleavage were purified on an Aquapore 1 mm × 10 cm OD-300 column with an Applied Biosystems (Foster City, CA) model 130A high-pressure liquid chromatography (HPLC) system.

The amino acid sequence of the peptides was determined by automated Edman degradation on an Applied Biosystems model 477A protein sequencer with a model 120A microbore HPLC system for on-line analysis of phenylthiohydantoin amino acids. An Applied Biosystems model 473A protein sequencer was also used for sequencing peptide fragments and data from this instrument was analyzed using the model 610A data analysis software version 1.2 (Applied Biosystems).

The mass of intact HB-GAM was determined by plasma desorption mass spectrometry with a Bio Ion 20 (Bio Ion Division, Applied Biosystems Inc., Uppsala, Sweden) time-of-flight mass spectrometer (Sundqvist *et al.*, 1984). A 200-pmol aliquot of HB-GAM was applied to aluminized Mylar (Bioion Division Applied Biosystems, Uppsala, Sweden) that was electrosprayed previously with 100 μg of nitrocellulose (Jonsson *et al.*, 1986). The sample disc was spin-dried and washed with 20 μl of 0.1% trifluoroacetic acid (Roepstorff *et al.*, 1987). Ionization of the protein was achieved by exposure to $^{252}Cf$ fission fragments (Sundqvist *et al.*, 1984). Spectra were collected for 20–24 h at 20 kV accelerating potential. Spectra were calibrated with hydrogen and sodium ions.

The number of free sulfhydryl groups was determined by incubating 200-pmol aliquots of native HB-GAM in 6 M guanidine-HCl 0.25 M Tris-HCl, pH 8.5, 1 mM EDTA in the presence of 440 mM 4-vinylpyridine for 90 min at room temperature. As a control experiment, HB-GAM was first reduced by incubation in the above buffer containing 50 mM 2-mercaptoethanol for 60 min at 37 °C and subsequently incubated with 4-vinyl-pyridine as above. The reaction mixtures were desalted on a 1 mm × 10 cm Aquapore OD-300 column and dried with a Speed Vac (Savant Instruments, Farmingdale, NY) concentrator. The amino acid compositions of the reduced/pyridylethylated and unreduced/pyridylethylated samples were determined after hydrolysis in 6 M HCl at 150° for 60 min. The hydrolyzed samples were derivitized with phenylisothiocyanate and the resulting PTC-amino acids were analyzed on a model 840 PICOTAG amino acid analysis system (Waters, Medford, MA).

### *Bioassays*

The ability of HB-GAM to stimulate DNA synthesis was determined using Balb/3T3 clone A31, NRK 49F (American Type Culture Collection, Rockville, MD), or BALB/MK (Weissman and Aaronson, 1983) cells. Either Balb/3T3 or NRK cells were grown to 50% confluence in 48-well cluster dishes containing Dulbecco's modified Eagle's medium (DMEM):F12 (1:1 mixture, Irvine Scientific, Santa Anna, CA) and 10% bovine calf serum (Inovar, Gaithersburg, MD) at 37°C, 5% $CO_2$ in a humidified atmosphere. The growth media was removed and replaced with starvation medium (DMEM:F12 1:1 mixture, 0.5% bovine calf serum, 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.2). The cells were incubated as above in this medium for an additional 72 h. Dilutions of HB-GAM or FGF-1 were added to the wells and incubated at 37°C for 16 h. The cells were labeled with 0.5 μCi/ml of $^3H$-methyl-thymidine (25 mCi/mmol, Amersham, Arlington Heights, IL) for an additional 4 h. Assays with BALB/MK cells were performed as previously described (Rubin *et al.*, 1989). The media was aspirated, and $^3H$-methyl-thymidine that had incorporated into DNA was precipitated by adding 100 μl of ice-cold 10% trichloroacetic acid to each well and incubating on ice for 15 min. DNA was solubilized with 1.0 M NaOH at 37°C for 15 min. The amount of radioactivity in each sample was determined by scintillation counting. All assays were performed in triplicate.

The human umbilical vein endothelial cells used in the growth assays were provided by T. Maciag (American Red Cross, Rockville, MD). The cells were maintained on fibronectin-coated tissue culture dishes in medium 199 (Irvine Scientific, Santa Ana, CA) containing 20% (vol/vol) fetal bovine serum, one times antibiotic-antimycotic (GIBCO, Grand Island, NY), 5 units/ml heparin (Upjohn, Kalamazoo, MI), 10 ng/ml human recombinant FGF-1, 5 μg/ml insulin, 4.8 ng/ml selenium, and 5 μg/ml transferrin. For the growth assays, the cells were trypsinized and seeded at $5.6 \times 10^4$ cells/well in 12-well cluster dishes (Corning, Silver Spring, MD) that were coated with fibronectin (5 μg/cm²) in the above media without FGF-1. The indicated amounts of HB-GAM or FGF-1 were added to wells in triplicate with or without 5 units/ml heparin. The medium and growth factors were changed every 2 d, and after 6 d the cells were trypsinized and counted with a hemacytometer.

The neurotrophic activity of HB-GAM was tested on dissociated chicken embryo cerebral cortical neurons (Kligman, 1982). Briefly, the cerebral cortex from four day-7 (stage 31–32) chicken embryos was dissected and treated with calcium-magnesium-free phosphate-buffered saline (PBS) and the cells were suspended by gentle trituration with a pasteur pipet and counted. Neurons were plated at a density of $1 \times 10^4$ cells/cm² on 35-mm tissue culture plates that had been untreated or coated previously with 0.25 ml of either 25 μg/ml of poly-L-lysine (Sigma, St. Louis, MO) or 8 μg/ml of HB-GAM dissolved in sterile water. The plates were rinsed with sterile distilled water three times before adding the cells. After 42 h incubation in serum free medium (Ham's F12 base, 5 μg/ml insulin, 5 μg/ml transferrin, 20 nM progesterone, [Sigma] 100 μM putrescine, 30 nM sodium selenite [Aldrich, Milwaukee, WI]) at 37°C the cells were fixed in 1% glutaraldehyde in PBS and counted on a Nikon (Garden City, NY) Diaphot inverted microscope with a 20X objective under phase contrast. Neurite outgrowth was assessed by counting 100–200 cells in four fields of each culture plate. All assays were performed in duplicate.

## RESULTS

The purification methods used here resulted in the recovery of relatively large quantities of intact HB-GAM from bovine brain and chicken heart and indicated its presence in adult tissues of neural and non-neural origin. The yield of HB-GAM from bovine brain was significantly higher when 250 mM NaCl was added to the homogenization buffer; the yield was 120 ng/g (wet wt) and 17 ng/g (wet wt) with or without added NaCl, respectively. HB-GAM was also isolated from chicken heart by a chloroform/methanol procedure (Sasaki *et al.*, 1989). The yield from this source was 360 ng/g (wet

wt). These yields are significantly higher relative to the yield of other heparin-binding growth factors isolated from these tissues but is lower than the amount of HB-GAM isolated from postnatal rat brain (Rauvala, 1989).

The apparent molecular weight of HB-GAM as estimated by SDS-PAGE is ~18 000 Da (Figure 1), and the amino acid composition indicated it has a high content of lysine and cysteine residues. The number of free sulfhydryl groups was determined after reaction of HB-GAM with 4-vinyl-pyridine. Analysis for the content of pyridylethylated cysteine residues after acid hydrolysis and derivitization with phenylisothiocyanate revealed 4 pyridylethylcysteine residues present in unreduced HB-GAM and 10 pyridylethylcysteine residues in reduced HB-GAM. Because HB-GAM contains 10 cysteine residues, these results indicate that 6 of the 10 cysteine residues have formed disulfide bonds.

In certain cases the generation of a truncated form of HB-GAM was observed during the course of its purification. The quantitative conversion of the 18 000-Da form to a 15 000-Da form (Figure 1) was observed if HB-GAM was batch eluted from heparin-Sepharose with 1.5 M NaCl, diluted into 50 mM phosphate buffer, pH 6.0, and subsequently purified by ion-exchange chromatography. This conversion did not occur if HB-GAM was either eluted from heparin-Sepharose with a NaCl gradient or if it was purified immediately by reversed-phase HPLC.

The complete amino acid sequence of bovine HB-GAM was established by a combination of amino terminal sequencing and sequencing of peptides generated by either enzymatic or chemical cleavage. From the sequences of overlapping peptide fragments it was determined that bovine HB-GAM consisted of 136 amino acids (Figure 2). The calculated mass of this protein was 15 289 Da, which is considerably less than the apparent molecular weight estimated from SDS-PAGE (Figure 1). To determine whether there were any major post-translational modifications to the protein and to verify that the derived sequence was indeed complete, the mass of HB-GAM was determined by plasma desorption time-of-flight mass spectrometry. The mass was determined to be 15 291 mass units on the basis of the $(M + 3H)^{3+}$ ion. Therefore, we conclude that HB-GAM has not undergone any major post-translational modifications that would have added significant mass to the protein and that the amino acid sequence shown in Figure 2 is complete.

The amino acid sequence of chicken HB-GAM was also determined as described above; comparison of the bovine and chicken sequences revealed seven amino acid substitutions and one amino acid deletion (Figure 2). Serine$^{121}$ was not present in the chicken HB-GAM; thus, this polypeptide consists of 135 amino acids. The substitution of serine$^{115}$ in the bovine sequence with a proline in the chicken sequence is consistent with the residue present in that position of the rat and human

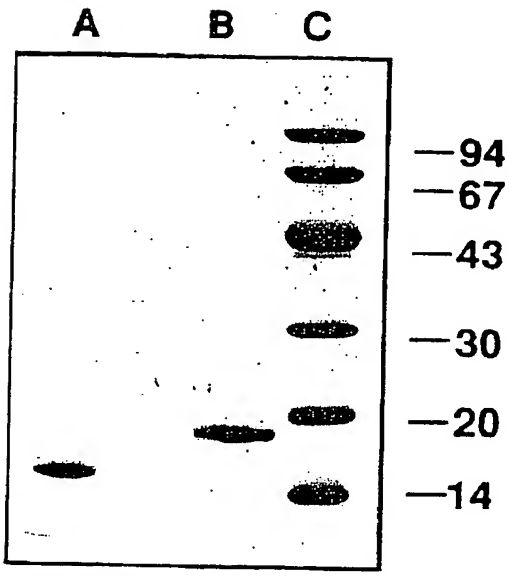


**Figure 1.** SDS-PAGE of intact and truncated HB-GAM. Samples were subjected to electrophoresis in a 13% acrylamide, 0.4% *N,N'*-methylenebisacrylamide gel polymerized in a Hoefer minigel apparatus with the buffer system of Laemmli (1970). Electrophoresis was carried out at 200 V until the marker dye reached the end of the gel. Lane A, truncated HB-GAM isolated by batch elution from heparin-Sepharose and ion-exchange chromatography; lane B, intact HB-GAM isolated by gradient elution from heparin-Sepharose and ion-exchange chromatography; lane C, Pharmacia low-molecular-weight standards. The numbers to the right indicate the approximate molecular weight $\times 10^{-3}$.

sequences of HB-GAM (Li *et al.*, 1990; Merenmies and Rauvala, 1990). It was not possible to precisely identify the residue at position 119 because the signal carry over from the previous cycle (which is a glutamine) interfered with determining whether the identity of residue 119 was truly a glutamic acid residue or a glutamine residue that had undergone substantial deamidation. The identity of residue 135 (the carboxyl-terminal residue of the chicken sequence) was determined by amino acid composition of the Glu-C-derived fragment (residues 128–135). The identity of the amino acid residue at position 135 could be either an asparagine or an aspartic acid because asparagine undergoes deamidation during acid hydrolysis.

As illustrated in Figure 3A, bovine HB-GAM in the presence or absence of heparin was not mitogenic for Balb/3T3 clone A31 cells. In contrast, FGF-1 in the presence of heparin elicits a potent mitogenic response with a half maximal dose of 0.3–0.5 ng/ml. Without heparin in the assay medium, FGF-1 is significantly less potent. Figure 3, B and C show the mitogenic response of Balb/MK (a mouse epidermal keratinocyte) and NRK (normal rat kidney fibroblasts), respectively, to FGF-1 and HB-GAM. The results of these assays further demonstrate the lack of mitogenic activity of HB-GAM. HB-GAM was also tested for its ability to promote the growth of human umbilical vein endothelial cells. As

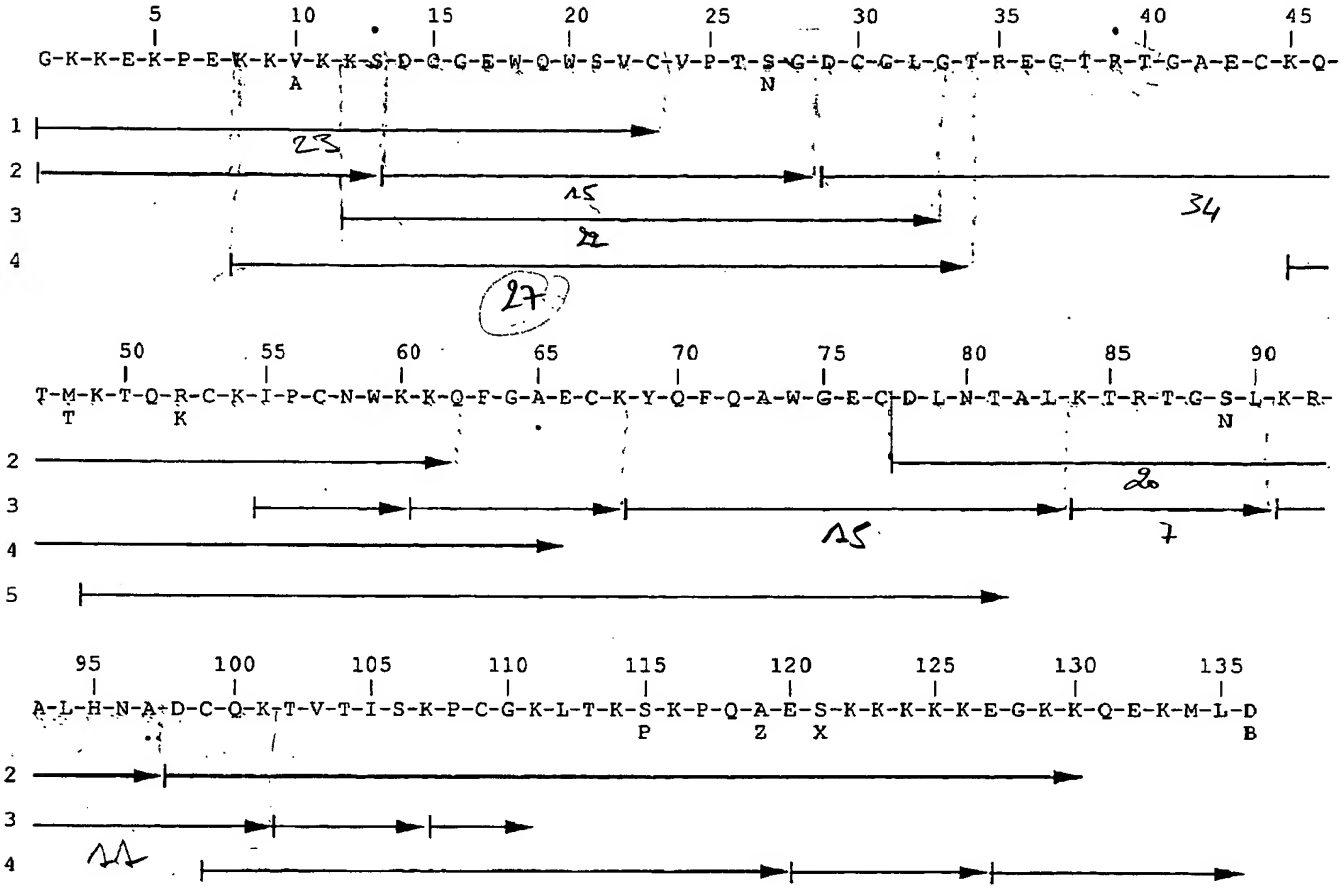

Figure 2. The amino acid sequence of bovine and chicken HB-GAM. The amino-terminal sequence of bovine HB-GAM and the sequences derived from cyanogen bromide and proteolytically derived fragments were aligned to give the complete 136 amino acid sequence. The amino acid sequence of chicken HB-GAM is listed under the bovine sequence with only the amino acid differences indicated. An X at position 121 indicates the residue that is deleted in the chicken sequence. The vertical lines indicate the beginning of the sequence of a fragment and the arrows indicate where sequencing of a fragment ended. The numbers to the left indicate the reagent used to generate the peptide fragments. 1, the amino-terminal sequence of intact HB-GAM; 2, endoproteinase Asp-N; 3, endoproteinase Lys-C; 4, endoproteinase Glu-C; 5, cyanogen bromide.

shown in Table 1, FGF-1 in the presence of heparin stimulated a 20-fold increase in cell number during the 6-d assay period. However, HB-GAM was not able to induce a growth response in the presence or absence of heparin. Therefore, HB-GAM (unlike FGF-1) is not a mitogen for fibroblasts, keratinocytes, or endothelial cells in vitro.

The ability of HB-GAM to act as a neurotrophic factor was tested using a chicken embryo cerebral cortical neurite outgrowth assay. When 500 ng/ml of HB-GAM was present in the assay medium and the cells were plated on untreated culture dishes, 3.6% of the neurons extended neurites (Figures 4A and 5A). There was a pronounced increase in neurite extension when the culture dish was coated with HB-GAM (Figures 4B and 5B); however, there was no significant increase in this response if in addition to coating the dish, HB-GAM was also added to the assay medium (Figures 4C and 5C). When poly-L-lysine was used as an attachment substrate and HB-GAM was added as a soluble factor, there was a small increase in neurite outgrowth compared with the untreated dish (Figures 4E and 5E). Figures 4D and 5D show the response to the negative control (poly-L-lysine coating with no soluble factor added) and Figures 4F and 5F show the response to the positive control (poly-L-lysine coating with 10 $\mu$l fetal bovine serum added as a soluble factor).

## DISCUSSION

In this report we describe the isolation and complete primary structure of HB-GAM, a highly basic heparin-binding protein from adult bovine brains or chicken hearts. Bovine HB-GAM consists of 136 amino acids; of these there are 10 cysteines and 28 lysines. Seven of the 10 cysteines are flanked by charged residues. The number of free cysteine residues in bovine HB-GAM was determined to be four, in contrast to the results reported previously (Kuo *et al.*, 1990). In that report $^{14}$C-iodoacetamide was incubated with HB-GAM for 10

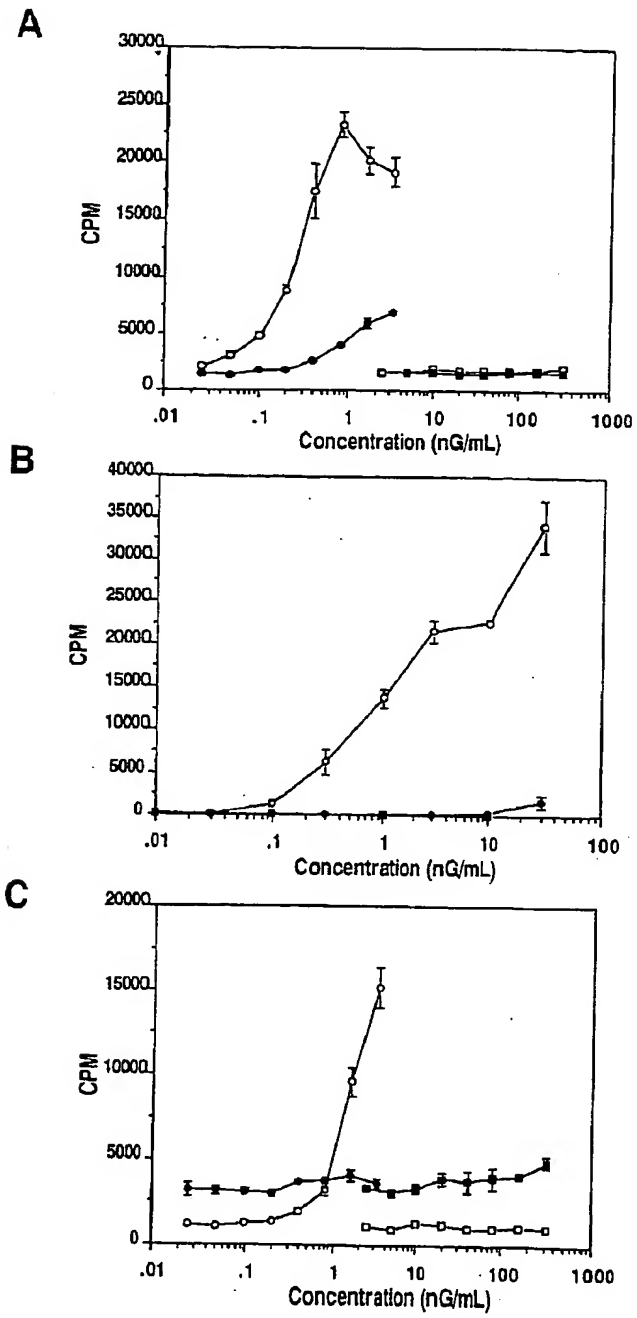


**Figure 3.** Mitogenic assay. Stimulation of DNA synthesis by HB-GAM or FGF-1. The magnitude of the response was measured by the amount of [$^3$H]thymidine that was incorporated into DNA in response to HB-GAM with 10 units/ml heparin □——□, or without heparin ■——■, and FGF-1 with heparin ○——○, or without heparin ●——●. (A) BALB/3T3 cells; (B) BALB/MK cells; (C) NRK cells.

min and no incorporation of radioactivity into the protein was observed. On the basis of those results, it was concluded that there are no free sulfhydryl groups in HB-GAM; however, the number of cysteine residues accessible to iodoacetamide after reduction was not determined. There are three potential nuclear targeting sequences based on the consensus sequence, K-R/K-X-R/K, as described previously (Chelsky *et al.*, 1989) and there are no consensus *N*-linked glycosylation sites. The mass of HB-GAM, determined by plasma desorption time-of-flight mass spectrometry, is in close agreement with the mass calculated from the amino acid sequence. Therefore, we conclude that there are no post-translational modifications to HB-GAM that would result in a significant increase in mass, and as suggested previously (Kuo *et al.*, 1990), the aberrant mobility of HB-GAM on SDS-PAGE is likely due to its inherent charge.

Brain is a rich source of heparin-binding growth factors and proteases. Any number of such proteins may copurify with HB-GAM resulting in preparations contaminated with mitogenic or proteolytic activities. Fur-

**Table 1.** Human umbilical vein endothelial cell growth assay.[a]

| | Growth factor concentration (ng/ml) | | | |
|---|---|---|---|---|
| | 0 | 1 | 10 | 100 |
| HB-GAM | 22 ± 22 | 21 ± 4 | 20 ± 2 | 16 ± |
| HB-GAM + 10 U/ml heparin | ND | 21 ± 8 | 17 ± 5 | 14 ± |
| FGF-1 + 10 U/ml heparin | ND | ND | 448 ± 33 | ND |

[a] Cell number/well × $10^{-3}$. Values are means ± SD. ND, not determined

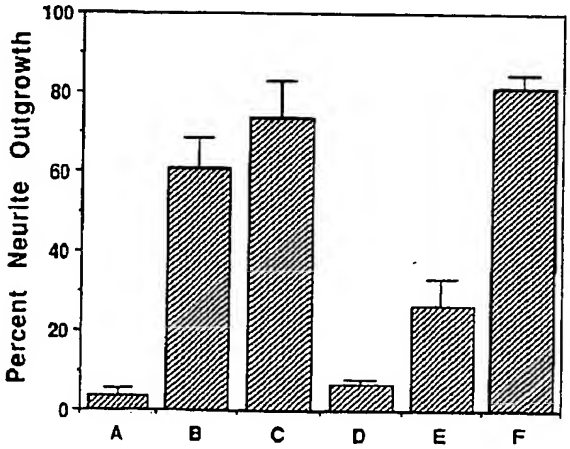


**Figure 4.** Neurite extension assay. Chicken cerebral cortex neurons were prepared from 7-d embryos (see MATERIALS AND METHODS) and plated on either HB-GAM coated, poly-L-lysine coated, or uncoated tissue culture dishes with or without HB-GAM or serum added to the culture medium. The cells were incubated at 37 μC for 42 h, fixed with 1% glutaraldehyde, and duplicate dishes scored. A positive response is a cell with at least one neurite that is twice the length of the diameter of the cell body. A, uncoated dish, 100 ng/ml HB-GAM in the medium; B, HB-GAM-coated dish, no addition to medium; C, same as B with 100 ng/ml HB-GAM in the medium; D, poly-lysine-coated dish, no addition to the medium; E, same as D with 100 ng/ml HB-GAM in the medium; F, same as D with 10% fetal calf serum in the medium.

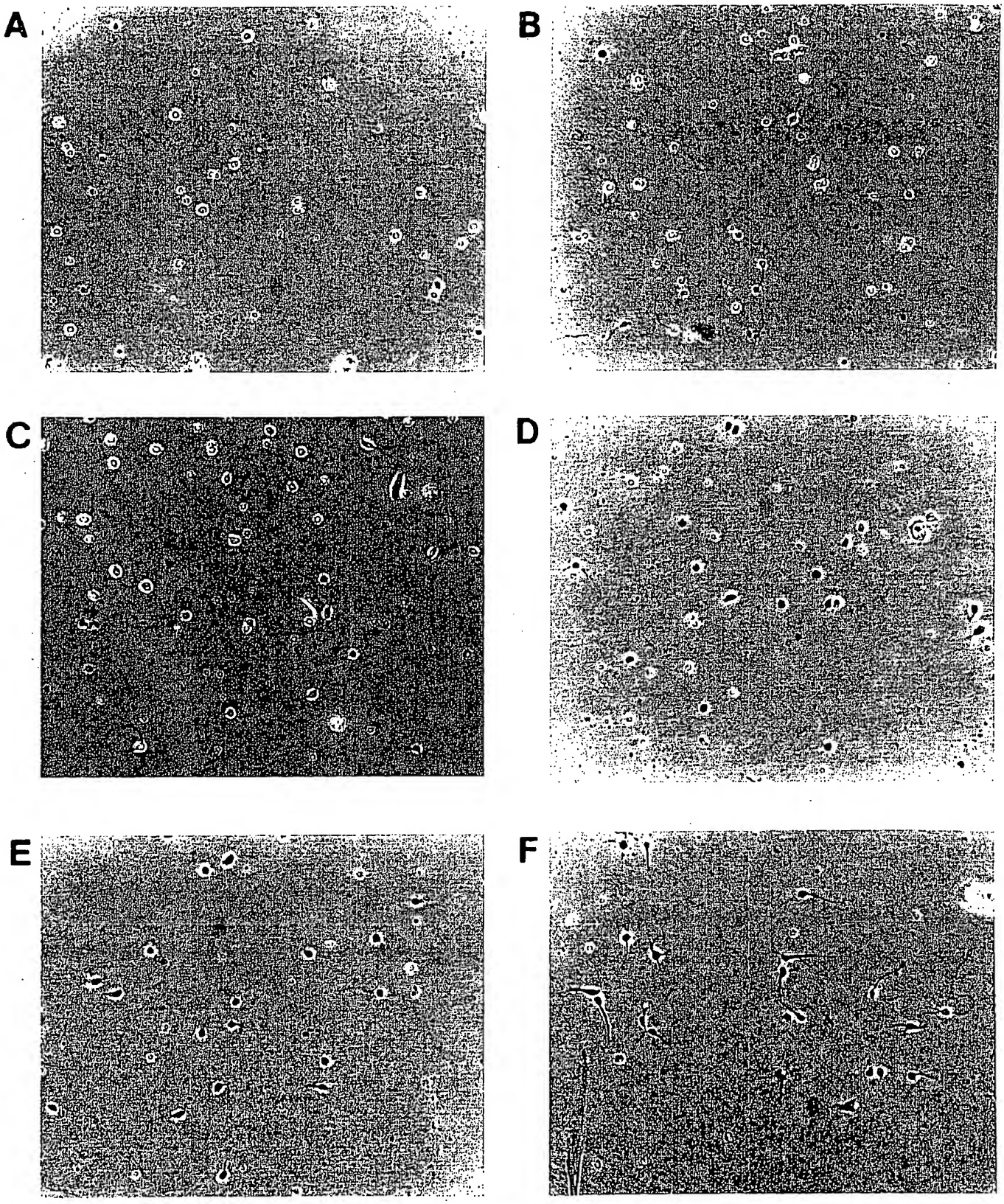


**Figure 5.** Photomicrographs of the neurite extension assay. The photomicrographs were taken at the time the assay was scored with a 20× objective on a Nikon Diaphot inverted phase contrast microscope. A–F are representative fields of the corresponding treatments described in Figure 4.

thermore, these activities may be increased or decreased by the pH of the buffers used during extraction and subsequent purification. We noted that under certain conditions used during the purification of HB-GAM that there was a nearly quantitative conversion of the full-length protein to a truncated form. It appears that this proteolytic activity has a different apparent affinity for heparin-Sepharose than HB-GAM because cleavage of HB-GAM was not observed if a salt gradient was used to elute the heparin-Sepharose rather than batch elution with 1.5 M NaCl. Therefore, HB-GAM does not appear to undergo additional processing other than cleavage of the signal peptide and the mature form of the bovine protein does consist of 136 amino acids. On the basis of two reports that describe the amino acid sequence of truncated HB-GAM, this cleavage can occur at two or three sites (Kuo *et al.*, 1990; Bohlen *et al.*, 1991). These cleavages remove a highly basic cluster of amino acids at the carboxyl-terminus. It was proposed that similar basic clusters of amino acids observed in the amino-terminal region were responsible for the heparin-binding activity of HB-GAM (Kuo *et al.*, 1990). However, we directly compared the heparin-binding activity of the truncated form of HB-GAM with that of the intact protein and found that they are eluted from heparin-Sepharose with the same concentration of NaCl (0.75–1.0 M). Therefore, this would indicate that the apparent affinity of HB-GAM for heparin may not be due solely to the basic clusters of amino acids found both at the amino- and carboxyl-termini of the protein.

The recovery of HB-GAM is increased by the addition of NaCl to the homogenization buffer. This result may be due to the disruption, by the increased ionic strength, of a complex formed between HB-GAM and an insoluble component of the tissue. However, HB-GAM did not bind to either gelatin-Sepharose or fibronectin-Sepharose (data not shown), a characteristic shared with a heparin-binding protein isolated from chick basement membranes (retinoic acid-induced heparin binding [RI-HB]) by extraction with a high ionic strength buffer (Vigny *et al.*, 1989; Urios *et al.*, 1991). In addition to these common characteristics, RI-HB has a similar apparent molecular weight, shares 50% amino acid sequence homology with HB-GAM, and is not a mitogen. RI-HB is widely expressed in early stages of embryogenesis and later becomes restricted to the lens capsule.

HB-GAM also shares a 50% amino acid sequence identity with MK, a protein expressed in mouse teratocarcinoma cells after treatment with retinoic acid (Kadomatsu *et al.*, 1988). Both proteins contain 10 cysteine residues and their relative positions are conserved. Like HB-GAM, MK has been shown to contain a signal peptide sequence and it has been demonstrated that MK is a secreted protein (Tomomura *et al.*, 1990). The tissue distribution of MK is ubiquitous during early embryogenesis beginning at day 5, but becomes more restricted after day 9 until its expression is observed mainly i the kidney (Kadomatsu *et al.*, 1990).

The tissue distribution of HB-GAM was determine by RNA and protein blotting techniques (Rauvali 1989). In 6-d postnatal rat tissues the HB-GAM mRN was abundant in brain and virtually undetectable i liver, spleen, kidney, and heart. The HB-GAM protei was detected by western blotting in brain, kidney, an to a lesser extent, in heart tissue. In adult rat tissues th HB-GAM mRNA and protein was detected in brain bu not in liver, spleen, kidney, or heart tissues. Althoug the level of expression of HB-GAM seems to be reduce in adult tissues, it is still relatively abundant. The evi dence presented here revealed higher quantities of HB GAM in chicken heart than was found in bovine brain

Comparison of the mitogenic activity of full-lengt HB-GAM with FGF-1 indicates it is relatively inactive At concentrations as high as 320 ng/ml, HB-GAM wa unable to stimulate DNA synthesis or proliferation o four different cell strains. However, full-length HB-GAM is active in a neurite extension assay. When used as an attachment substrate, HB-GAM is capable o stimulating near maximal neurite outgrowth in chicken embryo neurons within 42 h. It has been reported that the biological activity of HB-GAM is acid labile (Milner *et al.*, 1989). Because the purification scheme used to isolate HB-GAM for these experiments did not involve the use of detergent solubilization or organic solvents and acids such as those used in reversed-phase HPLC and resulted in apparently homogeneous preparations of full-length HB-GAM within 24 h, it is unlikely that our purification methods resulted in an acid-inactivated preparation of HB-GAM. Further studies to determine the biological activities of this protein in vivo are necessary before its precise function(s) can be established. Li *et al.* (1990) named this protein pleiotrophin to "reflect its diverse activities." The name HB-GAM seems more appropriate as it was the name given by Merenmies and Rauvala (1990) who were the first to determine the sequence of the cDNA encoding this protein.

The amino acid sequence of HB-GAM is highly conserved even among nonmammalian species. For example, there are only seven amino acid differences between the human and chicken sequences. Because the structure of HB-GAM has been conserved, the functional properties may be similar among diverse species. Its role as a neurotrophic factor (NTF) in vivo has not been established. The suggestion that this protein is indeed an NTF may be premature (Kovesdi *et al.*, 1990; Bohlen *et al.*, 1991). The following set of criteria provides a basis for the definition of an NTF (Walicke, 1989). These criteria are 1) a demonstration of specific receptors on neurons, 2) available in the normal neuronal environment, and 3) NTF effects in vivo as well as in vitro. Certainly the second criteria has been satisfied by Ravaula (1989); however, evidence for specific neuronal receptors for HB-GAM is lacking and evidence for in

vivo effects has not yet been established. Laminin and fibronectin both promote neurite extension but neither are considered to be an NTF. HB-GAM appears to function more like these extracellular matrix proteins rather than as a soluble NTF.

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# Dominant Negative Effectors of Heparin Affin Regulatory Peptide (HARP) Angiogenic and Transforming Activities*

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**Isabelle Bernard-Pierrot‡§¶, Jean Delbé‡§, Vincent Rouet‡, Marc Vigny‖, Marie-Emmanuelle Kerros‡, Danièle Caruelle‡, Daniel Raulais‖, Denis Barritault‡, José Courty‡**, and Pierre Emmanuel Milhiet‡ ‡‡**

*From the ‡Laboratoire de recherche sur la Croissance Cellulaire, la Réparation et la Régénération Tissulaires (CRRET), CNRS UPRES-A 7053, Université Paris XII, Avenue du Général de Gaulle, 94010 Créteil Cedex, France, the ‡‡Centre de Biochimie Structurale, UMR554 INSERM, 29 rue de Navacelles, 34090 Montpellier, France, and ‖INSERM U440, Université PARIS VI, rue du Fer à moulin, 75005 Paris, France*

**Heparin affin regulatory peptide (HARP) is an heparin-binding growth factor, highly expressed in several primary human tumors and considered as a rate-limiting angiogenic factor in tumor growth, invasion, and metastasis. Implication of this protein in carcinogenesis is linked to its mitogenic, angiogenic, and transforming activities. Recently, we have demonstrated that the C-terminal residues 111–136 of HARP are required for its mitogenic and transforming activities (Bernard-Pierrot, I., Delbe, J., Caruelle, D., Barritault, D., Courty, J., and Milhiet, P. E. (2001) *J. Biol. Chem.* 276, 12228–12234). In this paper, HARP deleted of its last 26 amino acids was shown to act as a dominant negative effector for its mitogenic, angiogenic, transforming, and tumor-formation activities by heterodimerizing with the wild type protein. Similarly, the synthetic corresponding peptide P111–136 displayed *in vitro* inhibition of wild type HARP activities, but in this case, the inhibition was mainly explained by the competition of the peptide with HARP for the binding to the extracellular domain of the high affinity ALK receptor.**

The expression of polypeptide growth factors is tightly regulated and contributes to the timely development of tissues during embryonic and neonatal growth. As depicted in several studies, gene expression of these growth factors is deregulated in solid tumors, and these polypeptides contribute to autocrine and paracrine stimuli leading to the development of the tumor tissues. Among these growth factors, angiogenic polypeptides appear to be very interesting therapeutic targets because angiogenesis plays a key role in cancer development, and negative regulators of angiogenesis are under clinical evaluation (reviewed in Ref. 1).

Heparin affin regulatory peptide (HARP)[1] (2), also known as pleiotrophin (3) or heparin-binding growth-associated molecule (4), is an 18-kDa-secreted polypeptide that constitutes with Midkine (MK), a two-member family among the heparin-binding growth factors. The pattern of expression of HARP suggests that this molecule has functions in neuronal migration and in epithelium-mesenchyme interactions during the embryonic development (5). However, although HARP is mainly down-regulated after birth, it seems to play a key role in cell growth and differentiation during adulthood. It seems particularly involved in tumor growth and angiogenesis (6). HARP mRNA and/or protein are highly expressed in neuroblastoma, glioblastoma, and gastrointestinal, prostate, and primary breast cancers (7, 8). They are also retrieved in tumor cell lines of different origins including pancreas (9), lung (10), and ovary (11). In pancreatic cancers, HARP expression by tumors is correlated with its elevated level in patient sera (8).

HARP displays several *in vitro* biological activities. It induces neurite-outgrowth in embryonic neural cells through its binding to the heparan sulfate proteoglycan *N*-syndecan or to the chondroitin sulfate proteoglycan RPTPβ (12, 13). In addition, HARP stimulates cellular proliferation of a wide range of cell types including fibroblast, endothelial, and epithelial cells (2, 7, 14). HARP also displays transforming activity because transfection of NIH-3T3 or normal rat kidney (NRK) fibroblastic cells with the HARP cDNA led to morphological transformations, anchorage independent growth, and tumor formation in nude mice (15). In agreement with its role in angiogenesis, HARP induces migration of aortic endothelial cells in collagen (16) and enhances plasminogen activator activity in the same cells (17). Recently, the mitogenic and transforming activities of HARP have been linked to its binding to the tyrosine kinase receptor anaplastic lymphoma kinase (ALK) inducing its phosphorylation and recruitment of downstream effector molecules such as IRS-1, Shc, phospholipase C-γ, and phosphatidylinositol 3-kinase (18).

NMR experiments have clearly demonstrated that the HARP molecule is organized into two β-sheet domains linked by a flexible linker, and this structure is maintained through five intrachain disulfide bonds (19). Each β-sheet domain contains a heparin-binding site, which plays a role in the modulation of HARP mitogenic activity (20). In addition, we have shown that at least one heparin-binding domain is involved in the dimerization of HARP (21). Two clusters of basic residues are located

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§ Both authors contributed equally to this work.

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** To whom correspondence should be addressed. Tel.: 33-1-45-17-17-97; Fax: 33-1-45-17-18-16; E-mail: Courty@univ-paris12.fr.

[1] The abbreviations used are: HARP, heparin affin regulatory peptide; MK, Midkine; ALK, anaplastic lymphoma kinase; FCS, fetal calf serum; MAP, mitogen-activated protein; WT, wild type; DMEM, Dulbecco's modified Eagle's medium; CAPS, 3-(cyclohexylamino)propanesulfonic acid; PBS, phosphate-buffered saline; PBS-T, PBS containing 0.05% Tween 20; BSA, bovine serum albumin; BEL, bovine epithelial lens cells; FGF, fibroblast growth factor; ERK, extracellular signal-regulated kinase.

at the N-terminal and C-terminal regions, and we have recently shown that the lysine-rich C-terminal tail of HARP is required for its mitogenic and tumor-formation activities but not for the neurite outgrowth-promoting activity (22). Indeed the deletion mutant HΔ111–136, deleted of its last 25 amino acids, was shown to display no mitogenic activity on BEL cells or NIH-3T3 cells and shown to be unable to induce tumor formation in nude mice but to stimulate the neurite outgrowth of rat embryonic neurons.

In this paper, we demonstrate for the first time that HΔ111–136 and the corresponding synthetic polypeptide P111–136, lacking mitogenic, angiogenic, and tumor formation activities, could act as potent inhibitors of these HARP biological activities.

## EXPERIMENTAL PROCEDURES

*Materials*—Culture medium, fetal calf serum (FCS), and G418 were supplied by Invitrogen. Superblocker® solution was purchased from Pierce, horseradish peroxidase-conjugated rabbit anti-goat, goat anti-rabbit immunoglobulins G was purchased from Jackson, and 3,3',5,5'-tetramethylbenzidine dihydrochloride substrate and disuccinimidyl suberate were purchased from Interchim (Montluçon, France). Goat anti-human HARP antibodies were from R&D (Oxon, United Kingdom). Heparin-Sepharose gel and Mono-S column were from Amersham Biosciences, Immobilon-P was from Millipore Corp. (Saint-Quentin en Yvelynes, France), BM chemiluminescence® and FuGENE6® were from Roche Mannheim (Meylan, France), and mouse anti-phospho-p44/p42 MAP kinase was from New England BioLabs (Saint-Quentin en Yvelynes, France). [Methyl-$^3$H]thymidine was provided by ICN (Orsay, France) and Matrigel was from BD PharMingen. Peptides P111–136 (KLTKPKPQAESKKKKKKEGKKQEKMLD) and P1–21 (AEAGKKEKPEKKVKKSDCGEW) were synthesized by the Synt:em laboratory (Nîmes, France). Recombinant fibroblast growth factor-2 (FGF-2), MK, and mutated and wild type (WT) HARP were purified in the laboratory by sequential heparin-Sepharose and Mono-S chromatographies from bacteria and conditioned media of eukaryotic cells, respectively (22). Bovine angiogenin was a generous gift from Dr. G. Spik (UMR8576 CNRS, Villeneuve d'Ascq, France).

*Thymidine Incorporation Assay and Phosphorylation of MAP Kinase*—The incorporation of [methyl-$^3$H]thymidine by serum-starved NIH-3T3 cells was performed as previously described (22). $2.5 \times 10^5$ NIH-3T3 cells were seeded in 35-mm culture dishes for 24 h, serum-starved for 24 h, and stimulated 5 min at 37 °C with samples. Cells were lysed with electrophoresis sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 0.02% bromphenol blue, 2% SDS, and 5% β-mercaptoethanol), and the presence of p42/p44 or phospho-p42/p44 MAP kinase was detected by Western blot (22).

*In Vitro Angiogenesis Assay*—Three-dimensional collagen gels were prepared following the procedure of Montesano with minor modifications (16, 23). Briefly, $0.9 \times 10^5$ aortic endothelial cells (ABAE) per well of a 4-well culture plate were seeded on a three-dimensional collagen gel in complete medium. 24 h after plating, proteins were added every day. Three days later, tubular network structures were quantified using phase contrast microscopy. The experiment was repeated three times and carried out in duplicate. Data are the means of 10 randomly chosen fields/well with indicated standard errors and representative results shown.

*In Vivo Mouse Matrigel Plug Assay*—Four Swiss mice per sample (Janvier, Le Genest St Isle, France) were subcutaneously injected with 0.2 ml of Matrigel alone or containing proteins according to the previously described procedure (24). The injected Matrigel rapidly formed a single solid gel plug. Mice were sacrificed after 6 days, and the Matrigel plugs were excised and frozen in liquid nitrogen. Sample sections of 10 μm thick were made using a cryostat (Leica) and extemporaneously stained with Gomori-Trichrome for microscopic observation. Endothelial cell invasion and vessel ingrowth was quantified by computer image analysis using the NIH Image analyzing software.

*DNA Transfection*—MDA-MB 231 cells were culture in DMEM containing 4.5 g/liter glucose supplemented with 10% FCS at 37 °C with 7% $CO_2$. MDA-MB 231 cells were transfected with 4 μg of pCDNA-3 or pCDNA3-HΔ111–136 plasmids using the liposomal system FuGENE6 according to the manufacturer's protocol and selected 48 h later with G418 at 600 μg/ml. Medium was changed every 2 days until colonies were formed. The amount of HARP proteins secreted by each clone was determined using an immunometric assay (25), and clones secreting the largest amount of HARP were selected. For these clones, the presence of both HARP and mutant proteins in the culture medium was further investigated by Western blotting after heparin-Sepharose purification.

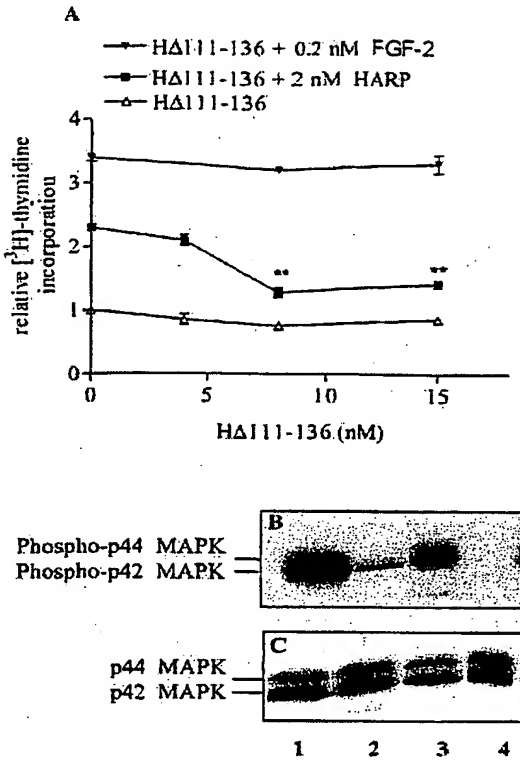


FIG. 1. **Inhibition of WT HARP mitogenic activity by HΔ111–136.** *A*, stimulation of [$^3$H]thymidine incorporation in serum-starved NIH-3T3 cells treated with various concentration of HΔ111–136 in the presence or absence of 2 nM HARP or 0.2 nM FGF-2. Results are the mean of three separate experiments carried out in triplicate, and the standard errors are indicated. **, $0.001 < p < 0.01$. *B* and *C*, lysate from NIH-3T3 control cells (*lane 2*) or from cells stimulated 5 min with 10% FCS (*lane 1*), 2 nM HARP (*lane 3*), 2 nM HΔ111–136 (*lane 4*), 2 nM HARP, and 2 or 6 nM HΔ111–136 (*lanes 5* and *6*, respectively) was separated on a 10% SDS-PAGE. ERKs and phosphorylated ERKs present in the lysate were detected by Western blotting using an antiphospho p42/p44 MAP kinase antibody (*B*) and an anti-p42/p44 MAP kinase antibody (*C*), respectively.

*Soft Agar Assay*—$2 \times 10^4$ cells of the clonally selected MDA-MB 231 cell line were seeded in triplicate into 6-well plates containing agar and DMEM supplemented with 10% FCS. After 14 days, colonies with diameters greater than 50 μm were scored as positive using a phase contrast microscope equipped with a measuring grid.

*Tumor Formation in Nude Mice*—Tumor formation in 5-week-old male athymic nude mice (Nu/Nu; IFFA CREDO Laboratories) was tested by subcutaneous injection of $4 \times 10^6$ transfected MDA-MB 231 cells suspended in 100 μl of DMEM in each flank. Tumor size was measured twice a week, starting from the second week following injection. Mice were sacrificed 6 weeks after injection.

*Chemical Cross-linking Experiment*—Purified recombinant HARP or HΔ111–136 (15 pmol) were pre-incubated for 60 min at 25 °C in the presence of 10 μg/ml heparin and treated with 0.5 mM disuccinimidyl suberate according to the manufacturer's protocol, and products were analyzed using a 15% acrylamide SDS-PAGE and anti-HARP Western blotting experiment.

*Construction and Production of ALK Extracellular Domain (RECA)*—A construct coding for the entire extracellular domain of human ALK (that we named RECA) linked to a $His_8$ tag was generated using PCR experiments from the pCDNA3-ALK (26). The resulting RECA-His cDNA was further subcloned into the pCEP4 vector (Invitrogen) to generate pCEP4-RECA-His. The human embryonic kidney HEK-293 cell line transfected with the EBNA-1 gene (Invitrogen) was cultured in DMEM containing 10% FCS and 0.4 mg/ml geneticin at 37 °C in 5% $CO_2$. HEK-293 cells plated at $5 \times 10^3$ cells/cm$^2$ for 2 days were transfected by electroporation with pCEP4-RECA-His. Thirty-six

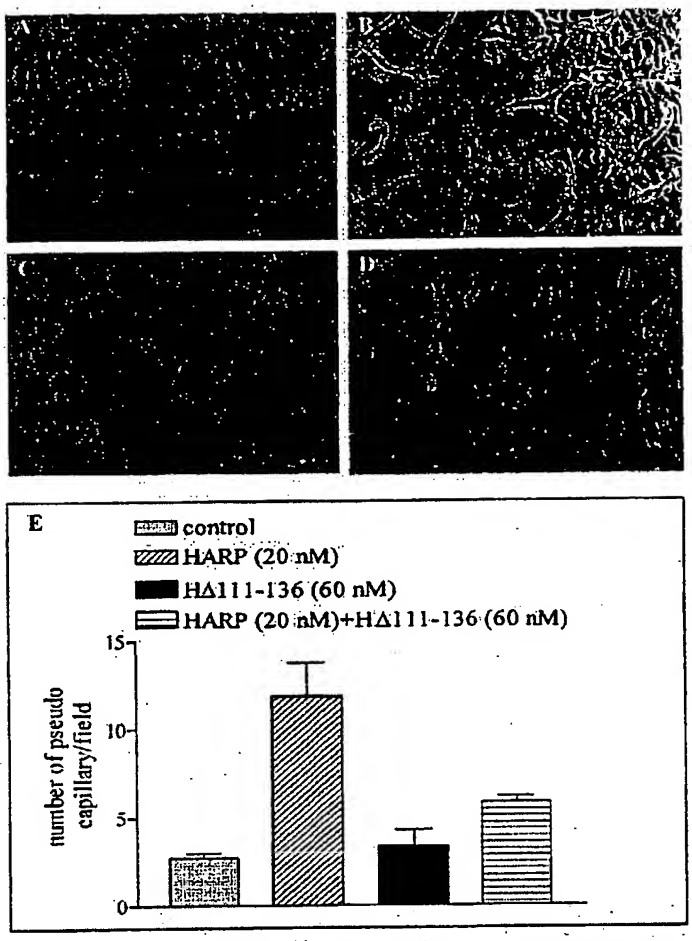


FIG. 2. **HΔ111–136 inhibition of HARP-induced *in vitro* angiogenic activity.** Aortic endothelial cells (ABAE) were seeded on a three-dimensional collagen gel in complete medium. 24 h after plating, 20 nM proteins were added every day, and 3 days later tubular network structures were quantified using phase contrast microscopies (×100) (*E*). *A–D* represent phase contrast micrographs obtained in the absence of recombinant protein (*A*) or in the presence of 20 nM HARP (*B*), 60 nM HΔ111–136 (*C*), or a mixture of both WT and mutant HARP proteins (*D*).

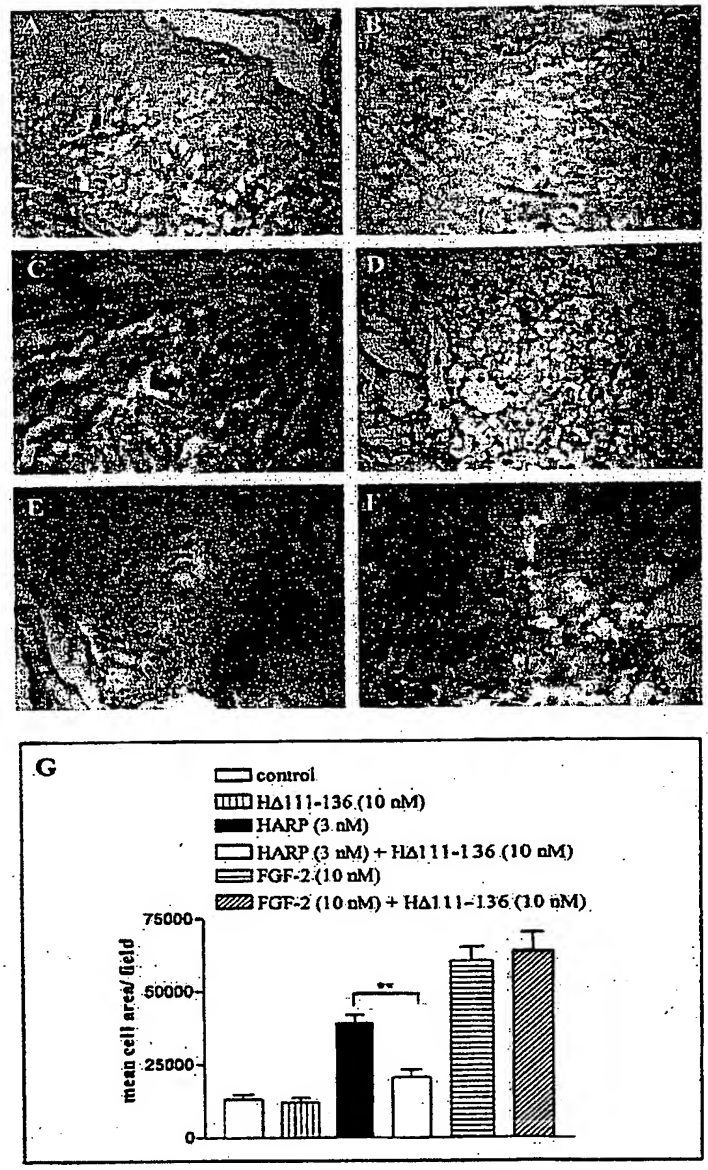


FIG. 3. **HΔ111–136 inhibition of HARP-induced *in vivo* angiogenic activity.** Liquid Matrigel was injected subcutaneously into mice alone or in the presence of proteins. Mice were sacrificed after 6 days, and Matrigel plugs were sectioned and stained using the Gomori-Trichrome method for microscopic observation (magnification ×100). The number of infiltrated endothelial cells was determined by averaging six fields from one section of Matrigel plugs per mouse (*G*). The results are expressed as the mean of four mice per condition, and standard errors are indicated (**, $0.001 < p < 0.01$). Micrographs *A–F* represent Gomori-Trichrome staining of a sectioned Matrigel plug alone (*A*) or in the presence of 10 nM HΔ111–136 (*B*), 3 nM HARP (*C*), a 3 nM HARP/10 nM HΔ111–136 mixture (*D*), 10 nM FGF-2 (*E*), or a 10 nM FGF-2/10 nM HΔ111–136 mixture (*F*).

hours after transfection, medium was changed and hygromycin (Sigma) was added to the medium at 0.5 mg/ml. After 10 days of selection, the medium was changed to the serum-free AIM-V synthetic medium (Invitrogen). The AIM-V production media were collected every 2–3 days. The secreted RECA-His protein was purified from the AIM-V production media over a nickel-nitrilotriacetic acid superflow column (Qiagen, Courtaboeuf, France) following the manufacturer's protocol. Rabbits were immunized with the purified RECA-His protein. IgG fractions of the immune sera were immunopurified using DEAE chromatography.

*Cell-free RECA/HARP Binding Studies*—Purified recombinant HARP, HΔ111–136, FGF-2, MK, angiogenin, peptides P111–136 and P1–21 were coated 1 h at 37 °C in 10 mM CAPS, pH 11, (100 μl) on a 96-well EIA plate (costar). Nonspecific binding sites were blocked 1 h at 37 °C with PBS, 0.05% Tween 20 (v/v) (PBS-T) containing 3% BSA (w/v). Wells were then incubated with RECA diluted in PBS-T containing 1% BSA for 1 h at 37 °C and exposed for 1 h at 37 °C to rabbit anti-RECA antibodies diluted at 1 μg/ml in PBS-T containing 1% BSA. Bound antibodies were visualized using a peroxidase-labeled goat anti-rabbit antibodies at a 1:5000 dilution in PBS-T containing 1% BSA, and the peroxidase activity was detected with 3,3′,5,5′-tetramethyl benzidine dihydrochloride substrate according to the supplier.

## RESULTS

*Inhibition of HARP Mitogenic Activity by HΔ111–136*—The mitogenic activity of HARP in the presence of different concentrations of the HΔ111–136 mutant was investigated on serum-starved NIH-3T3 cells. A 2-fold increase of [$^3$H]thymidine incorporation was observed for 2 nM HARP and inhibited in a dose-dependant manner by addition of HΔ 111–136 (Fig. 1*A*). A maximum 70% inhibitory effect was observed for 7.5 nM HΔ111–136 corresponding to a molecular ratio of 3:1 between mutant and HARP protein. As expected from previous results (22), no mitogenic activity of HΔ111–136 alone was observed. In a control experiment, stimulation of [$^3$H]thymidine incorporation induced by 0.2 nM FGF-2 was not affected by HΔ111–136 (Fig. 1*A*). The specificity of the HΔ111–136 inhibitory effect was also tested with the structurally related molecule MK, which displayed no

TABLE I

*HΔ111–136 reverses colony formation in soft agar of MDA-MB 231 cells and prevents tumor formation in the nude mice*

MDA-MB 231 cells were transfected with pCDNA3-HΔ111–136 or pCDNA3 alone as a control. G418-resistant clones that expressed high levels of recombinant mutant HARP protein (HΔ111–136, clones 1 and 2) and those transfected with the vector alone (control 1 and 2) were selected. $2 \times 10^4$ cells/plate were seeded in triplicate into 6-well plates in soft agar. After 14 days, colonies with a diameter greater than 50 μm were scored as positive. Results are mean of two different experiments. One control and one HΔ111–136-expressing clone were used to inject six nude mice using $4 \times 10^6$ cells/flank. 6 weeks after injection of cells, mice were weighted, tumors were measured, and the average of the volume of tumors was expressed in cubed millimeters ($mm^3$).

| Stably transfected MDA-MB 231 cells | Number of colonies/$cm^2$ in soft agar assay | Number of mice with tumor | Tumor size | Weight |
|---|---|---|---|---|
| | | | $mm^3$ | g |
| Control-1 | 190 ± 10 | 10/12 | 51 ± 12 | 23 ± 2 |
| Control-2 | 160 ± 8 | | | |
| HΔ111–136 clone1 | 78 ± 7 | 1/12 | 2 | 31 ± 1 |
| HΔ111–136 clone2 | 87 ± 10 | | | |

effects upon 2 nM HARP stimulation (data not shown). Because the HARP mitogenic signal, at least in BEL and NIH-3T3 cells, is transduced through the MAP kinase signaling pathway leading to the phosphorylation of ERK1 and ERK2 (22, 27), the ability of HARP to induce MAP kinase phosphorylation in the presence of HΔ111–136 was evaluated (Fig. 1*B*). As expected from cell proliferation experiments ERK1 and ERK2 phosphorylation induced by HARP was inhibited in a dose-dependant manner by the addition of HΔ111–136.

*Inhibition of in Vitro and in Vivo HARP Angiogenic Activity by HΔ111–136*—As the HΔ111–136 mutant protein specifically inhibited the HARP-induced cell proliferation, its effect on the angiogenic activity of HARP was investigated *in vitro* and *in vivo* using aortic bovine endothelial cells cultured on a three-dimensional collagen gel (23) and a mouse Matrigel plug assay (24), respectively. *In vitro*, 20 nM HARP induced a tubular network structure formation within 72 h (Fig. 2*B*). As compared with the HARP-treated cells (Fig. 2*A*), 60 nM HΔ111–136 inhibited the HARP-induced angiogenic effect by more than 66% (Fig. 2*E*), whereas no significant effect was observed either in cells treated with 60 nM HΔ111–136 alone (Fig. 2*C*) or in control cells (Fig. 2*A*). A tubular network comparable with that formed using HARP was also obtained with 3 nM FGF-2, and no significant effect was observed with HΔ111–136 (data not shown). In the *in vivo* angiogenesis assay, a 3-fold increase in the infiltration of endothelial cells was observed when 3 nM HARP was added to Matrigel as compared with control (Matrigel alone) (Fig. 3, *A*, *C*, and *G*). Addition of 10 nM HΔ111–136 with 3 nM HARP in Matrigel, corresponding to a 3:1 ratio, resulted in a 50% inhibition of the angiogenic activity of HARP, whereas no Matrigel infiltration was observed with HΔ111–136 alone (Fig. 3, *B*, *D*, and *G*). As a control, 10 nM FGF-2 was tested and a more pronounced increase, as compared with HARP, of the infiltration of Matrigel by endothelial cells was obtained. As expected from the *in vitro* assay, no significant inhibition of FGF-2 by HΔ111–136 was observed (Fig. 3, *E*, *F*, and *G*).

*In Vitro and in Vivo Inhibition of HARP-transforming Activity by HΔ111–136*—The human breast cancer cell line MDA-MB 231 has been previously demonstrated to produce endogenous HARP, which acts in an autocrine manner. These cells were therefore used to test the inhibitory effect of the HΔ111–136 mutant protein on HARP-transforming activity. MDA-MB 231 cells were transfected with pCDNA3 alone (control) or pCDNA3-HΔ111–136, and clones were selected for their ability to secrete HARP. The presence of both HARP and HΔ111–136 proteins in the culture media was checked by Western blotting experiments, and proteins were similarly secreted (data not shown). Two representative clones (control and HΔ111–136-expressing cells) were further studied for their ability to grow in soft agar. Under our experimental conditions, HΔ111–136-expressing clones formed 2-fold fewer colonies than control cells (Table I). These results suggested that HΔ111–136 had inhibited the anchorage-independent growth of MDA-MB 231 cells. To further investigate the inhibitory effect of HΔ111–136 protein on HARP-transforming activity, one HΔ111–136 clone and one control clone were tested for their ability to form tumors in nude mice. Six weeks after the injection, ten of twelve mice injected with MDA-MB 231 control cells had developed tumors, whereas only one mouse injected with MDA-MB 231 cells expressing HΔ111–136 had developed a tiny tumor (Table I). A difference of weight between the two pools of mice could also be noticed. To ensure that tumors derived from G418-resistant-injected cells, tumor fractions were dispersed and cultured in medium containing 600 μg/ml of the antibiotic. Under these conditions, most cells from the tumors appeared to be G418-resistant (data not shown). These results indicate that the ability of MDA-MB 231 cells to form tumors in nude mice was strongly inhibited by a dominant negative effect of HΔ111–136 mutant protein and that the transforming activity of these cells is really dependent on their HARP expression.

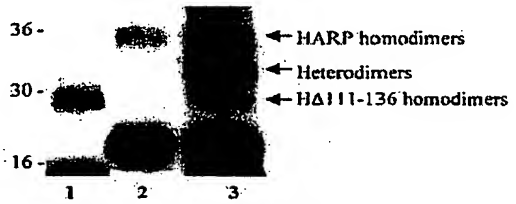


FIG. 4. **Heterodimerization of WT HARP with HΔ111–136.** 15 pmol of purified HARP (*lane 2*), HΔ111–136 (*lane 1*), or a mixture of both proteins (*lane 3*) were preincubated with 10 μg/ml heparin and treated with the cross-linking reagent disuccinimidyl suberate. Proteins were immunoblotted with goat anti-human HARP antibodies after SDS-PAGE separation under reducing conditions and transferred. Molecular mass markers are given in kDa.

*Molecular Mechanisms Involved in the Dominant Negative Effect of HΔ111–136*—Two hypothesis could be raised to explain the dominant negative effect: i) because HARP had been demonstrated to homodimerize (21), HΔ111–136 could form a non-functional heterodimer with HARP and ii) the mutant protein could compete against HARP for binding to the high affinity receptor ALK and consequently block signal transduction.

To determine whether HΔ111–136 was capable of heterodimerizing with HARP, we performed chemical cross-linking and Western blotting experiments. As expected, HARP could form homodimers in the presence of heparin (Fig. 4, *lane 2*) (21), and similarly, HΔ111–136 could also homodimerize (Fig. 4, *lane 1*). However, in the presence of WT and mutant proteins, both homo- and heterodimers were observed (Fig. 4, *lane 3*).

The ability of HΔ111–136 to bind to the high affinity ALK

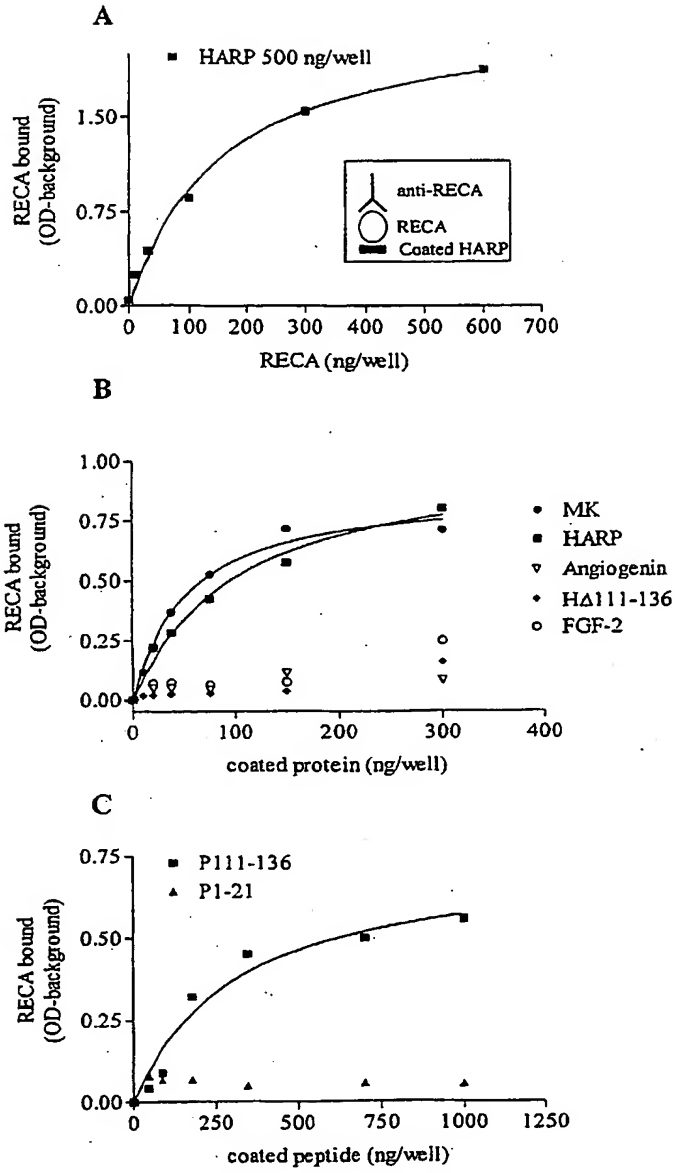


FIG. 5. **Cell-free RECA/HARP binding assay.** *A*, 500 ng/well of purified recombinant WT HARP were coated on an enzyme immunoassay plate and incubated with increasing amounts of the recombinant extracellular domain of the ALK receptor (RECA). RECA binding was estimated using immunodetection. The *inset* illustrates the experimental setup. *B* and *C*, various quantities of HARP, HΔ111–136, FGF-2, MK, angiogenin (*B*), or peptides P111–136 and P1–21 (*C*) were coated and incubated with 300 ng/well of RECA detected as in *A*. Each experiment was carried out three times in triplicate, and representative results are shown.

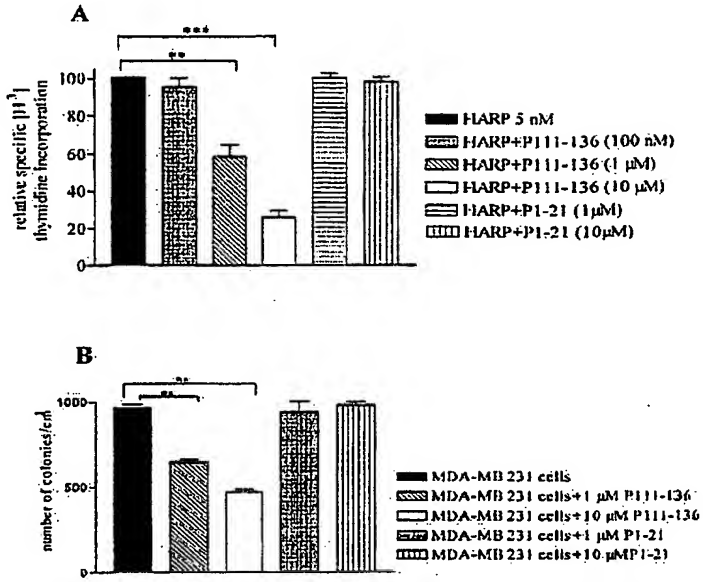


FIG. 6. **Inhibition of HARP biological activities by P111–136.** *A*, [$^3$H]thymidine incorporation of serum-starved NIH-3T3 cells treated with 5 nM HARP in the presence of various concentrations of P111–136 or P1–21. The results are the means of three separate experiments carried out in triplicate, and the standard errors are indicated: **, $0.001 < p < 0.01$; ***, $p < 0.001$. *B*, inhibition of HARP-transforming activity by P111–136. $3 \times 10^4$ MDA-MB 231 cells were seeded in triplicate into 6-well plates in soft agar containing various amount of P111–136 or P1–21. After 12 days, colonies with a diameter greater than 50 μm were scored as positive. Experiments were repeated two times, and representative results are shown. Standard errors are indicated: **, $0.001 < p < 0.01$.

receptor was investigated by enzyme-linked immunosorbent assay using the recombinant extracellular domain of the ALK receptor (RECA) produced in high eukaryotic cells. Recombinant purified HARP (500 ng/well) was coated and incubated with increasing concentrations of RECA ranging from 0 to 600 ng/well (Fig. 5*A*). Anti-RECA immunodetection revealed that RECA binds HARP in a dose-dependant manner reaching a plateau at a RECA concentration of 300 ng/well. Then, various quantities of either HARP, HΔ111–136, or control basic proteins including MK, FGF-2, and angiogenin were coated and incubated with 300 ng of RECA per well (Fig. 5*B*). RECA displayed a specific and saturable binding to the precoated HARP and MK proteins, whereas only background signals were detected with the HΔ111–136 mutant protein, FGF-2, or angiogenin. These results strongly suggest the absence of HΔ111–136 binding to its high affinity receptor and could explain the absence of signal transduction using this mutant (Fig. 1*B*, *lane 4*) (22).

*Inhibition of WT HARP in Vitro Biological Activities by the P111–136 Peptide*—Taken together, these results and our previous report seem to implicate the lysine rich C-terminal domain of HARP in the binding to its high affinity receptor ALK. To confirm this hypothesis, the ability of the peptide P111–136, corresponding to the last 25 amino acids of HARP, to interact with RECA was tested using the enzyme-linked immunosorbent assay described above. The P1–21 peptide was used as a control because it also contained a lot of lysines and has a similar backbone size. Various amounts of P111–136 and of the P1–21 N-terminal peptide of HARP (28) were coated and incubated with 300 ng of RECA per well (Fig. 5*C*). A specific and saturable signal was observed when P111–136 was immobilized, whereas only a background signal was detected using P1–21. Our results strongly strengthened the fact that HARP binds to the ALK receptor via its C-terminal domain and suggested that P111–136 could act as a dominant negative effector to inhibit WT HARP biological activities. This assumption was validated because P111–136 inhibited, in a dose-dependant manner, the [$^3$H]thymidine incorporation induced by HARP (Fig. 6*A*). About 75% inhibition was observed on serum-starved NIH 3T3 cells with 10 μM P111–136, although P1–21 was unable to inhibit HARP stimulation. Inhibition of the *in vitro* transforming activity of HARP by P111–136 was also evaluated in soft agar assays using MDA-MB 231 cells. Cells were seeded in soft agar and various amounts of P1–21 and P111–136 were added in culture medium. As shown in Fig. 6*B*, the transforming activity of WT HARP was prevented in a dose-dependant

manner by addition of P111–136, and no inhibition was observed with P1–21. The number of colonies was 50% decreased by 10 $\mu$M P111–136 peptide.

## DISCUSSION

HARP is expressed in many human tumors and tumoral cell lines including neuroblastoma, glioblastoma, melanoma, pancreatic, and breast cancers (6–10) and can be an *in vivo* rate-limiting angiogenic factor in tumor growth and metastasis (29, 30). According to these studies, this molecule is now considered as an interesting target in cancer therapy (9, 29). Recently, we have demonstrated the involvement of the C-terminal 111–136 amino acid of HARP in the mitogenic and in the transforming activities of this growth factor. These results prompted us to investigate the potential dominant negative effect of the C-terminal-truncated protein for the different characterized biological activities of HARP.

*Dominant Negative Effect of HΔ111–136*—Inhibition of HARP mitogenic, transforming, and angiogenic activities was observed in the presence of HΔ111–136 suggesting that similar structural determinants of the HARP molecule were implicated in these three activities. The dominant negative effect of HΔ111–136 on HARP-transforming activity was investigated using MDA-MB 231 cells. These cells were previously shown to express HARP and its high affinity receptor ALK, which is essential for the malignant phenotype (31, 32). *In vitro*, stable overexpression of the HΔ111–136 mutant protein by the MDA-MB 231 cell line resulted in a 50% decrease of colony formation in soft agar assay suggesting that the anchorage-independent growth was only partially due to HARP and that other growth factors are implicated. Similar partial inhibition had already been observed with other tumoral cell lines such as colorectal Colo 357 cells and melanoma WI 852 or 1205 Lu cells using HARP ribozyme targeting or HARP antisense strategies (9, 30, 33). However, *in vivo*, tumor formation into nude mice, usually obtained with MDA-MB 231 cells, was completely abolished when HΔ111–136-expressing MDA-MB 231 cells were injected suggesting that both the growth advantage and the angiogenic activity were inhibited. The dominant negative effect of HΔ111–136 on angiogenic activity was also supported by its ability to *in vitro* inhibit the formation of capillary network in a three-dimensional collagen gel and to strongly decrease *in vivo* the HARP-induced infiltration of Matrigel plugs by endothelial cells.

*Molecular Mechanism Involved in the Dominant Negative Effect*—The dimerization of growth factors such as FGF-1 and FGF-2 is responsible for receptor dimerization, cellular activation, and cell proliferation (34), and such a mechanism has been proposed for HARP (21). In regards to the results presented here, it seems that heterodimerization of the C-terminal mutant with HARP mainly explains its dominant negative effect. This heterodimerization is favored for a 3:1 molecular ratio between HΔ111–136 and HARP suggesting that under these conditions an excess of the mutant protein can dissociate HARP homodimers, inducing the formation of non-functional heterodimers. A similar mechanism had been proposed for HARP-transforming activities (35). These results strengthen the role of HARP dimers as the active forms of HARP for its mitogenic, angiogenic, and transforming activities.

During this study, we also investigated the binding of HΔ111–136 and HARP to the extracellular domain (RECA) of the tyrosine kinase receptor ALK, recently identified as a part of the HARP signaling pathway involved in mitogenic and transforming activities. The involvement of ALK in the HARP biological activities that we evaluated was first attested by the ability of RECA to bind HARP using the mitogenic activity assay with NIH-3T3 cells. RECA therefore induced a dose-dependant inhibition of the [$^3$H]thymidine incorporation due to HARP and transfection of non-responsive Chinese hamster ovary cells with the ALK cDNA-induced [$^3$H]thymidine incorporation and cellular proliferation of the selected clones by HARP (data not shown). The absence of interaction between HΔ111–136 and RECA that we have observed strongly suggests that HARP binds to ALK via its C-terminal domain. This assumption was reinforced by the binding of RECA to the synthetic peptide corresponding to the truncated mutant HΔ111–136 and the dominant negative effects of this peptide for HARP *in vitro* biological activities. The specificity of the interaction between the C-terminal part of HARP and RECA was mainly validated by the fact that P1–21, the HARP N-terminal peptide with basic properties close to P111–136 (pI of 9.37 *versus* 10.08), was not able to interact with RECA. Correlatively, a mutant deleted of the basic N-terminal cluster of lysines (HΔ1–12) kept mitogenic, angiogenic, and transforming activities and a binding capacity to RECA (data not shown). During these experiments, specific binding of MK to RECA was also observed, suggesting that ALK could be a common receptor for MK and HARP as observed with PTP$\zeta$ (36). This hypothesis is currently under study.

In conclusion, we have demonstrated the dominant negative effects on HARP biological activities of the truncated mutant HΔ111–136 and of the corresponding synthetic peptide P111–136. We have also underlined the binding of HARP to the high affinity receptor ALK through its C-terminal part. It is noteworthy that proteolysis of the C-terminal tail of HARP could be achieved *in vitro* by plasmin,[2] suggesting that such a mechanism could exist *in vivo* and participate in the regulation of HARP biological activities. Considering the importance of HARP as a rate-limiting autocrine growth factor in pancreatic and breast cancer cells (9, 29, 35) and its low expression in normal adult tissues, HARP appears as a promising target for cancer therapy. Synthetic HARP-related peptides or controlled expression of dominant negative mutant proteins into target cells could be used to block HARP growth tumor-promoting activity and now have to be evaluated.

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# HARP Induces Angiogenesis *in Vivo* and *in Vitro*: Implication of N or C Terminal Peptides

E. Papadimitriou,* A. Polykratis,† J. Courty,‡ P. Koolwijk,§ M. Heroult,‡ and P. Katsoris†,[1]

*Laboratory of Molecular Pharmacology, Department of Pharmacy, and †Division of Genetics, Cell and Developmental Biology, Department of Biology, University of Patras, Patras, Greece; ‡Laboratory of CRRET, University of Paris Val de Marne, Avenue du Général de Gaulle, 94010 Créteil, France; and §Gaubius Laboratory TNO-PG, Zernikedreef 9, 2333 CK Leiden, The Netherlands

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**HARP (heparin affin regulatory peptide) is a growth factor displaying high affinity for heparin. In the present work, we studied the ability of human recombinant HARP as well as its two terminal peptides (HARP residues 1–21 and residues 121–139) to promote angiogenesis. HARP stimulates endothelial cell tube formation on matrigel, collagen and fibrin gels, stimulates endothelial cell migration and induces angiogenesis in the *in vivo* chicken embryo chorioallantoic membrane assay. The two HARP peptides seem to be involved in most of the angiogenic effects of HARP. They both stimulate *in vivo* angiogenesis and *in vitro* endothelial cell migration and tube formation on matrigel. We conclude that HARP has an angiogenic activity when applied exogenously in several *in vitro* and *in vivo* models of angiogenesis and its $NH_2$ and COOH termini seem to play an important role.** © 2001 Academic Press

*Key Words:* **HARP; angiogenesis; endothelial cells; chicken embryo chorioallantoic membrane.**

HARP (heparin affin regulatory peptide), also called pleiotrophin or HB-GAM (heparin binding-growth associated molecule), is a 18 kDa secreted protein with distinct lysine-rich clusters within both the $NH_2$- and COOH-terminal domains (1). HARP has a high affinity for heparin and is localised in the extracellular matrix through interactions with glycosaminoglycans (2–5). It is highly conserved among species (2, 4, 6) and shares 50% homology with midkine and the avian retinoic-induced heparin binding protein (2, 7, 8). These proteins constitute a new heparin-binding growth factor family, structurally distinct from the FGF family (8).

[1] To whom correspondence should be addressed at Division of Genetics, Cell and Developmental Biology, Department of Biology, University of Patras, Patras, GR26504 Greece. Fax: 30-61-994797. E-mail: katsopan@upatras.gr.

HARP is a developmentally regulated protein that exhibits neurite outgrowth action in neonatal rat brain cells (1, 2, 9). Concerning its mitogenic activity, controversial results are reported, that vary according to the type and the origin of the studied cells (10–14). HARP was initially purified from bovine uterus (15) and neonatal rat brain (1). It is expressed in developing tissues (1, 16) and displays important functions in the growth and differentiation processes. In adults, HARP has been found in neuronal tissues, heart, uterus, cartilage and bone (17, 18), indicating that it may as well have important physiological roles during adulthood. HARP may also be an important regulator of tumour transformation. It is detected in various carcinomas (human breast and prostate cancer, neuroblastomas, benign meningiomas, small cell lung cancer, rat mammary tumours), exhibiting a protooncogene function (13, 19). It is constitutively expressed in cell lines derived from these tumours and is involved in tumour growth and metastasis (19–21). Cells transformed by the HARP cDNA, when implanted into nude mice, form highly vascularised tumours. Moreover, many of the HARP expressing tumours become more invasive when HARP gene is overexpressed (17) and reduction of HARP levels reduces the number of blood vessels in primary tumours, indicating a possible action of HARP related to the blood vessel network formation of solid tumour mass (21).

We have recently demonstrated a mitogenic effect of human recombinant HARP expressed in bacterial cells when presented to endothelial cells as a substrate and this effect was mimicked by two peptides corresponding to its $NH_2$ and COOH termini (22). In the present study, we investigated the effect of the above mentioned HARP and peptides on angiogenesis, using a variety of *in vitro* and *in vivo* assays.

## MATERIALS AND METHODS

*Cell culture.* Human umbilical vein endothelial cells (HUVEC), rat adrenal medulla microvascular endothelial (RAME) cells, bovine

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retinal endothelial cells (BREC), bovine brain capillary (BBC) endothelial cells and human foreskin microvascular endothelial cells (hMVEC) were isolated and cultured as previously described (10, 22, 23). Cultures were maintained at 37 °C, 5% $CO_2$ and 100% humidity.

*Purification of human recombinant HARP and HARP peptides.* Expression of the recombinant HARP was induced in *E. coli* BL21 pLys cells transformed with the human HARP-pETHH8 plasmid (kindly provided by P. Bohlen), as previously described (22). The bacterial product, similarly to the mammalian HARP, forms dimmers and oligomers that are stable in the presence of β-mercaptoethanol or dithiothreitol (data not shown). The formation of these covalent complexes is induced by transglutaminase, similarly to what has been described for midkine (24).

The sequences of the HARP peptides corresponding to the $NH_2$ and COOH terminus of the protein were $NH_2$-AEAGKKEKPEKKVKKSDCGEW-COOH (HARP residues 1–21) and $NH_2$-AESKKKKKEGKKQEKMLD-COOH (HARP residues 121–139) respectively, as previously described (22) and were obtained from (SYNT:EM, France).

*Matrigel tube formation assay.* The tube formation assay was performed as previously described (25). Briefly, matrigel was used to coat the wells of 24-well plates (0.25 ml/well) and was left to polymerise for 1 h at 37 °C. After polymerisation, 40,000 cells suspended in 1 ml of the corresponding medium supplemented with FCS (2% for HUVEC and BBC cells and 10% for RAME cells, that wouldn't form any tubes at lower serum concentration) were added to each well. HARP or the peptides were added to the corresponding wells just prior to addition of the cells. After 18 h of incubation (except for RAME cells that were incubated only for 6 h because of tube regression at longer incubations), the medium was removed, the cells were fixed and the length of the tube network was measured as previously described (25).

*Collagen tube forming assay.* Three dimensional type I collagen gels were prepared as previously described, by mixing 8 vol of a solution of rat collagen type I (4 mg/ml) with 1 vol of DMEM 10× and 1 vol of sodium bicarbonate (2.2 g/l) at 4 °C to prevent immediate gelation. 0.5 ml of the cold mixture was added to each well of a 24-well plate and allowed to gelify for 10 min at 37 °C (26). $5 \times 10^4$ BREC or $2 \times 10^4$ BBC cells were seeded on each collagen gel of a 24-well plate in DMEM supplemented with 2% (BREC) or 10% (BBC cells) FCS and 1 ng/ml FGF-2. One day after reaching confluency, different concentrations of HARP or each one of the peptides were added to the cell culture medium. The reorganisation of the endothelial cell monolayer was monitored and photographed with an Olympus IMT-2 inverted phase contrast microscope equipped with a digital camera.

*Fibrin tube forming assay.* Human fibrin matrices were prepared as described (23). Highly confluent endothelial cells were detached and seeded in a 1.25:1 split ratio on the fibrin matrices and cultured for 24 h in M199 medium supplemented with 10% human serum, 10% NBCS, and penicillin/streptomycin. After 24 h, the medium was replaced with medium containing 100 ng/ml HARP, 10 ng/ml FGF-2, 50 ng/ml vascular endothelial growth factor A (VEGF-A), 5 ng/ml tumour necrosis factor (TNF), or combinations of these mediators and cultured for a period of 7–9 days. The culture medium with the mediators was refreshed every two or three days. Invading cells and the formation of tubular structures of endothelial cells in the three-dimensional fibrin matrix were analysed by phase contrast microscopy. The total length of tube-like structures of six randomly chosen microscopic fields/well (7.3 $mm^2$/field = 44% of the well) was measured using an Olympus CK2 microscope equipped with a monochrome CCD camera (MX5) connected to a computer with Optimas image analysis software.

*Boyden chamber assay.* Migration assays were performed as previously described (27), in a 24-well microchemotaxis chamber (Costar), using untreated polycarbonate membranes with 8 μm pores. HUVEC, BBC and RAME cells were harvested and resuspended at a concentration of $10^5$ cells/0.1 ml, in the corresponding medium containing 0.25% bovine serum albumin (BSA). The bottom chamber was filled with 0.6 ml of the corresponding medium containing 0.25% BSA and HARP or each of the peptides. The upper chamber was loaded with $10^5$ cells and incubated for 4 h at 37 °C. FGF-2 was used as a positive control at a concentration of 5 ng/ml. After completion of the incubation, the filters were fixed with saline-buffered formalin and stained using DiffQuick. The cells that migrated through the filter were quantitated by counting the whole area of each filter using a grid and an Optech microscope at a 20× magnification.

*The chicken embryo chorioallantoic membrane (CAM) assay.* The *in vivo* CAM angiogenesis model (28) was used with minor modifications. Leghorn fertilised eggs (Pindos, Greece) were incubated for 4 days at 37 °C, when a window was opened on the egg shell, exposing the CAM. The window was covered with tape and the eggs were returned to the incubator until day 9, when the test molecules were applied. HARP or the peptides were added as a solution containing different amounts of the agents in a final volume of 20 μl of PBS, at day 9 of chicken embryo development on an area of 1 $cm^2$ of the CAM, restricted by a plastic ring. In a different group of eggs, which was used as control, only PBS was applied. After 48 h of incubation at 37 °C, the CAMs were fixed *in situ* with saline-buffered formalin, excised from the eggs, placed on slides and left to air-dry. Pictures at a 2.5× magnification were then taken through a stereoscope equipped with a digital camera and the total length of the vessels was measured as described above for the matrigel tube formation assay. Assays for each test sample were carried out three times and each experiment contained 10–20 eggs per data point.

*Statistical analysis.* The significance of variability between the results from various groups was determined by one-way analysis of variance (ANOVA). Each experiment included triplicate measurements for each condition tested, unless otherwise indicated. All results are expressed as mean ± SEM from at least three independent experiments.

## RESULTS

The ability of human recombinant HARP produced in bacterial cells and both peptides corresponding to its terminal domains to stimulate tube formation *in vitro* was tested using three different assays of angiogenesis, namely the collagen gel (26), the matrigel (25) and the fibrin gel (23).

*HARP stimulates tube formation on collagen gels.* When seeded on collagen gels, BBC endothelial cells formed a monolayer (Fig. 1, top, A). In the presence of HARP, there was a complete change of phenotype and the cells formed cord-like structures within 24 h, which were observed on and at several levels under the monolayer (Fig. 1, top, B). The formation of cord-like structures seemed to be concentration-dependent, starting at 10 ng/ml of HARP and reaching a plateau between 100 and 300 ng/ml. The effect was more intense when HARP was added in the mass of the collagen gel instead of being added in the cell culture medium. When either the $NH_2$ or the COOH peptide was added to the cell culture medium, there was only a weak stimulation, observed after several days (data not shown).

Human recombinant HARP added to the cell culture medium of BREC stimulated cord-like structures formation in a concentration-dependent way, with the

maximum effect observed between 100 and 300 ng/ml (Fig. 1, top, C and D, and bottom).

RAME cells or HUVEC plated on collagen gels did not survive and HARP did not improve their survival.

*HARP stimulates tube formation on matrigel.* The ability of HARP or either of the two peptides to promote tube formation was also tested on matrigel, which measures both migration and differentiation of the cells (25). BBC endothelial cells on matrigel did not form an extensive tubular network when cultured in low serum medium (Fig. 3A). HARP stimulated tube formation by BBC endothelial cells in a concentration-dependent manner, with a maximum effect observed at 300 ng/ml (Figs. 2A and 3A and 3B). Both HARP peptides stimulated tube formation by these cells in a way similar to the intact molecule (data not shown). On the contrary, HARP or each peptide had a small inhibitory effect on HUVEC tube formation, which was not statistically significant (Figs. 2B and 3C and 3D). In RAME cells, HARP or the peptides stimulated tube formation in a concentration-dependent manner, with maximal effect at 100 ng/ml (Figs. 2C and 3E and 3F).

*HARP stimulates tube formation on fibrin gels.* To investigate the ability of HARP or its terminal peptides to induce endothelial cell tube formation on fibrin gels, hMVEC were cultured on top of three-dimensional fibrin matrices. In this *in vitro* model, the combination of a growth factor (e.g. FGF-2 or VEGF-A) with the inflammatory mediator TNF$\alpha$ induces tube formation by the hMVEC (23), whereas these mediators by themselves are not effective (data not shown, ref. (23). HARP, either in the absence or presence of TNF$\alpha$ did not induce tube formation. However, HARP was found to have a marginally significant potentiating effect on FGF-2/TNF$\alpha$ or VEGF/TNF$\alpha$ induced tube formation in a concentration-dependent manner and the maximum effect was obtained with 100 ng/ml of HARP. There was an increase of 1.7 $\pm$ 0.29 folds (four independent experiments, $P < 0.05$) and 1.5 $\pm$ 0.35 folds (three independent experiments) in VEGF/TNF$\alpha$- and FGF-2/TNF$\alpha$-induced tube formation respectively, by the addition of HARP. Neither of the two terminal peptides of HARP had any effect on tube formation in this model, either alone or in combination with the other compounds (data not shown).

*HARP stimulates RAME cell migration.* The Boyden chamber test was used to estimate the migratory effect of HARP and the two peptides. As a positive control, FGF-2 was used at a concentration of 5 ng/ml (data not shown). All three molecules significantly induced migration of BBC (Fig. 4A) and RAME cells (Fig. 4B). HUVEC migration was not significantly affected by HARP or any of its peptides (Fig. 4C).

*HARP induces angiogenesis in vivo.* The chicken embryo CAM was used as a suitable *in vivo* model to

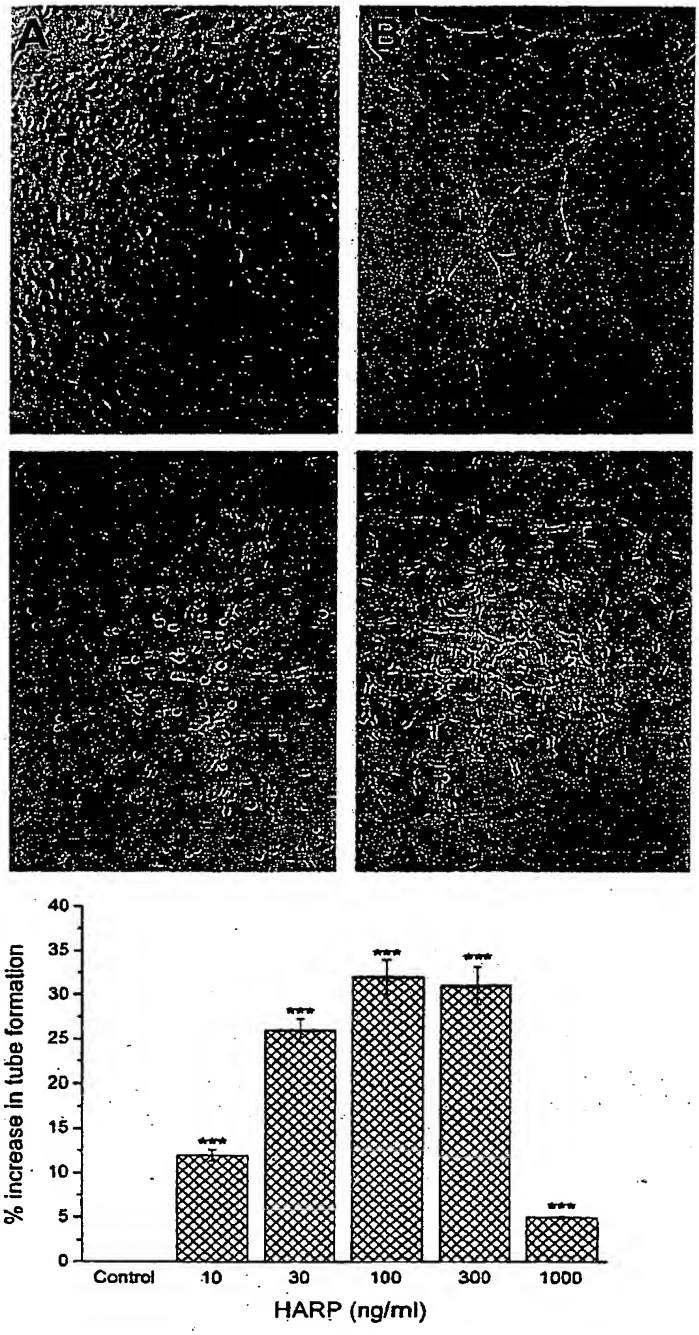


FIG. 1. (Top) Effect of human recombinant HARP on endothelial cells grown on collagen gels for 24 h (phase contrast microscopy). (A) Control BBC cells, (B) BBC cells treated with human recombinant HARP (100 ng/ml), (C) Control BREC, and (D) BREC treated with human recombinant HARP (100 ng/ml). Bar represents 500 $\mu$m. (Bottom) Effect of human recombinant HARP on BREC grown on collagen gels. Tube formation was assayed in the presence of varying amounts of HARP, as described under Materials and Methods. Total tube length was expressed as percent of the values obtained without stimulation. Data are the mean $\pm$ SEM of five independent experiments. ***$P < 0.001$.

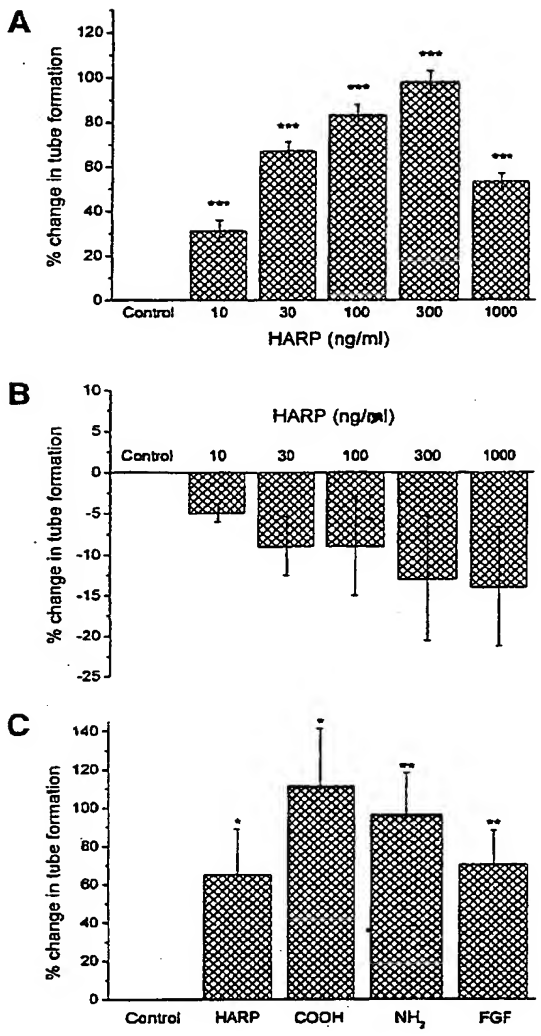


**FIG. 2.** Effect of human recombinant HARP on BBC cells (A) and HUVEC (B) grown on matrigel. Tube formation was assayed in the presence of varying amounts of HARP, as described under Materials and Methods. (C) Effect of human recombinant HARP and two peptides corresponding to its COOH- and $NH_2$-termini (100 ng/ml), on RAME cells grown on matrigel. Total tube length was expressed as percent of the values obtained without stimulation. Data are the mean ± SEM of three independent experiments. $^{***}P < 0.001$.

study the angiogenic action of HARP and its terminal peptides. Different concentrations of molecules were tested and 48 h after effector molecule application, the total length of the vessel network was measured, as described under Materials and Methods. As can be seen in Fig. 5, HARP and the COOH peptide induced angiogenesis in a dose-dependent and statistically significant manner. The $NH_2$ peptide also had a weak angiogenic effect which is statistically significant at a dose of 0.5 $\mu$g/ring. These results indicate that HARP is a potent angiogenic factor *in vivo* and its $NH_2$ and COOH termini may participate in this biological action.

## DISCUSSION

In the present study, we investigated the effect of human recombinant HARP expressed in bacterial cells and two synthetic peptides (HARP residues 1–21 and 121–139) on angiogenesis, using *in vitro* and *in vivo* experimental models. HARP has been shown to be

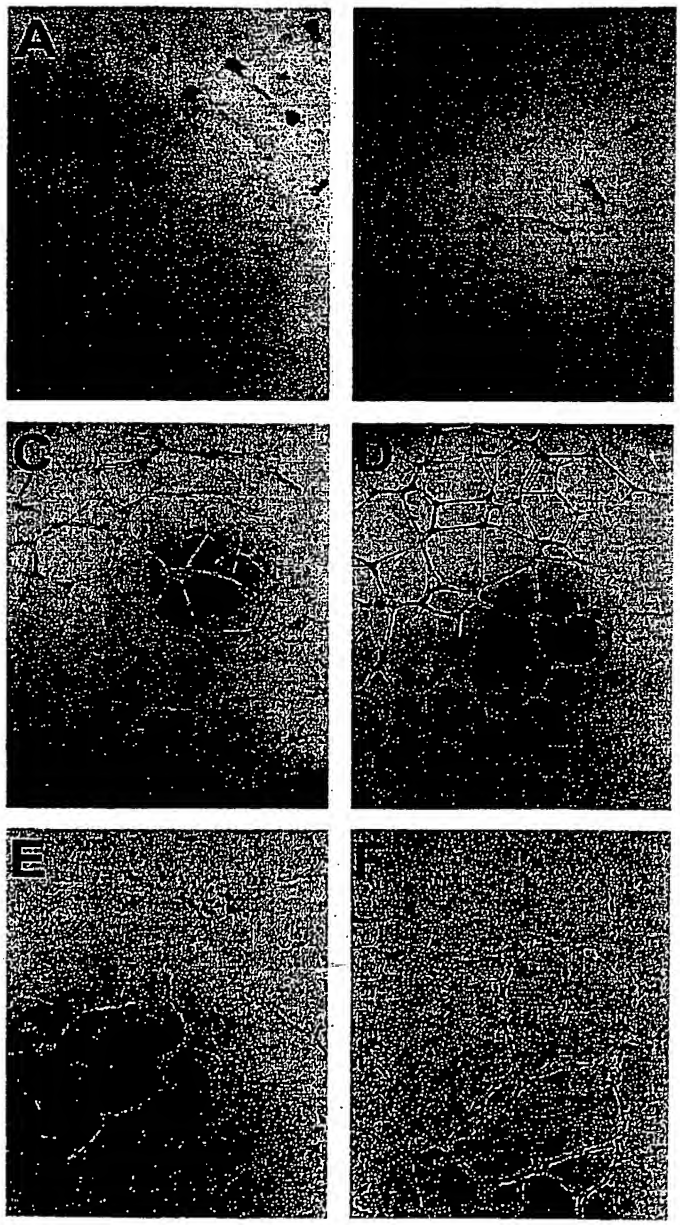


**FIG. 3.** Effect of human recombinant HARP on tube formation on matrigel (phase contrast microscopy). (A) Control BBC cells, (B) BBC cells treated with human recombinant HARP (100 ng/ml), (C) control HUVEC, and (D) HUVEC treated with human recombinant HARP (100 ng/ml). (E) Control RAME cells, (F) RAME cells treated with human recombinant HARP (100 ng/ml).

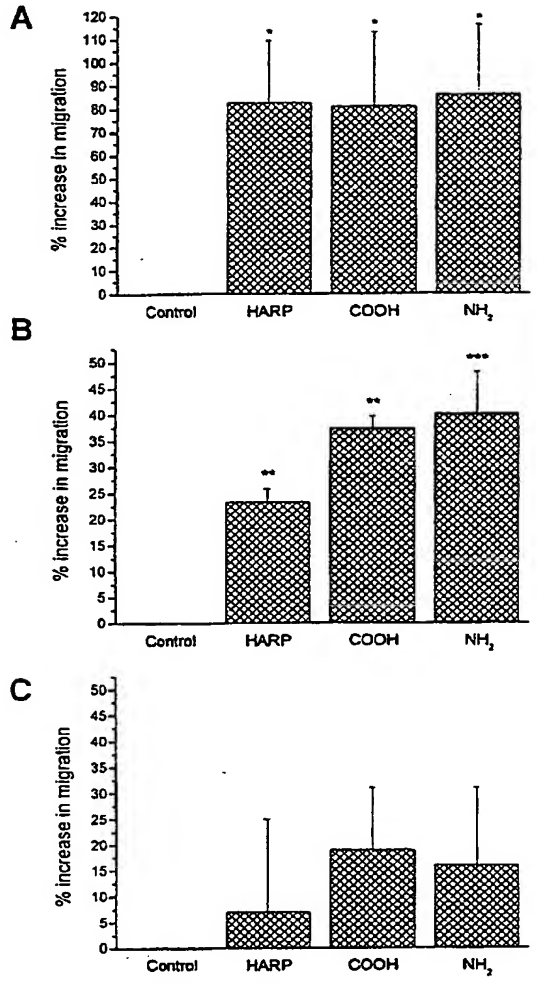


FIG. 4. Human recombinant HARP (100 ng/ml) and two peptides, corresponding to its COOH- and $NH_2$-termini (100 ng/ml), stimulate migration of BBC (A) and RAME cells (B) but not HUVEC (C). Results are expressed as percent of the values obtained without stimulation. FGF-2, which was used as a positive control, caused a 140% stimulation of migration (data not shown). Data are the mean ± SEM of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

expressed in a variety of primary human tumours (13) and has been correlated with enhanced tumour growth and vascular density (21, 29). The notion that HARP was angiogenic has been previously relied on an increased vascularity of tumors that expressed HARP (21, 29), the ability of human recombinant HARP expressed in a eucaryotic system to induce bovine aortic endothelial cell tube formation on collagen gels (26), and the angiogenic activity of HARP transfectant MCF-7 cells in the rabbit corneal assay (29). However, there was no direct and extensive evidence that HARP itself is an angiogenic molecule. Angiogenesis implicates many different actions, such as cell adhesion, migration, proliferation, differentiation and degradation of the basal lamina. In order to demonstrate the possible angiogenic role of HARP, we tested the implication of HARP as well as its two terminal peptides in endothelial cell processes involved in angiogenesis.

We have recently demonstrated that human recombinant HARP expressed in bacterial cells acts as a mitogen for different endothelial cells, namely BBC, RAME and HUVEC, when presented to them as a substrate (22). The response of these cells to both HARP and its terminal peptides is different, with HUVEC being the less responsive (22 and present study). Different cell surface molecules may interact with HARP among the different cells, depending on their origin and function. HARP could not be detected either in the culture medium or in the extracellular matrix of HUVEC (22). Other large vessel endothelial cells, like those deriving from bovine aorta, secrete HARP in both the culture medium and the extracellular matrix (data not shown) and are responsive to stimulation by HARP (26).

HARP binds with high affinity to heparin and to glycosaminoglycans, including heparan sulphate (4, 5) and dermatan sulphate (5) derived from extracellular matrix, functioning possibly as a mitogenic molecule entrapped in the extracellular space. In the same line, it had a greater tube formation activity when incorporated into the collagen gels than when added in the medium of the cells, while when matrigel was used as an *in vitro* angiogenesis assay, the effect was the same whether HARP was in the medium or in the mass of matrigel (data not shown). The difference could be due to the different nature of the *in vitro* assays used to test the implication of HARP on endothelial cell tube formation. On collagen gels, the cells can attach, spread, proliferate and finally form tubes (26), while on matrigel they do not proliferate, but migrate and finally differentiate forming tubes (25). Moreover, matrigel contains by itself several growth factors and other constituents of the extracellular matrix, while collagen or fibrin gels do not. It seems that HARP stimulates tube formation in all of these systems, however through different mechanisms. This is further strengthened by the fact that the two peptides (HARP residues 1–21 and 121–139) have a stimulating effect on matrigel but no effect on collagen or fibrin gel assays. The two lysine-rich terminal domains of HARP are implicated in multiple biological actions, since they stimulate endothelial cell proliferation (22) and are required for successful transformation of NIH 3T3 cells by HARP residues 41–64 (30). In addition, we have recently showed that the C-terminal part of HARP (HARP residues 111–136) is clearly involved in both mitogenic and cellular transformation effect (31). Both terminal regions account for the ability of HARP to bind tightly to heparin and to extracellular matrix (2, 4), although it was recently suggested that binding to heparin occurs primarily to the β-sheet domains of HARP (32). It

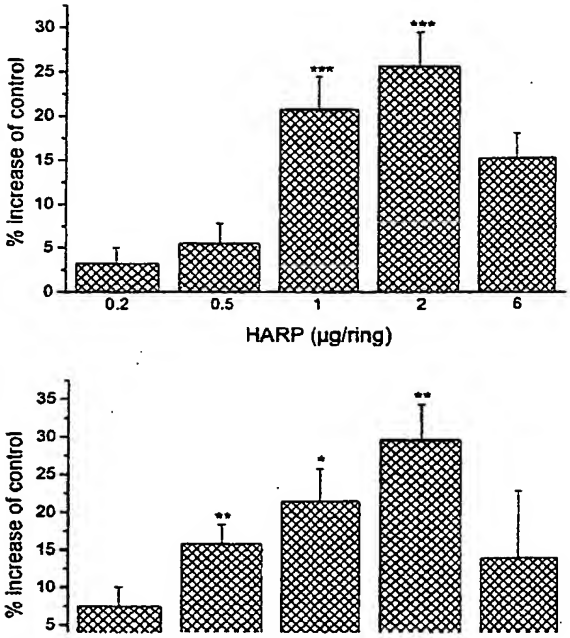


**FIG. 5.** Induction of angiogenesis by human recombinant HARP and two peptides, corresponding to its COOH- and $NH_2$-termini, in the chicken embryo CAM assay. (Top) Different quantities of tested molecules in the same final volume (20 $\mu$l) were applied on an area of 1 $cm^2$, restricted by a plastic ring, at CAMs of day 9, as described under Materials and Methods. After 48 h of incubation at 37 °C, the CAMs were fixed, excised from the eggs, and photographed and the total length of capillaries network was measured. Data are the mean ± SEM of three independent experiments, each one with 20 eggs tested for each concentration used. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (Bottom) Representative pictures showing the CAM vascular network when unstimulated or stimulated with 2 $\mu$g of HARP. Bar represents 500 $\mu$m.

has been hypothesised that the positively charged $NH_2$ and COOH termini of HARP interact with the receptor or other cell surface molecules and facilitate HARP binding to a high affinity receptor (30).

In the present study, both HARP peptides had by themselves a significant effect, similar to the effect of HARP and in some cases seem to be active even at lower amounts than the whole molecule. As we have recently reported, HARP can form covalently bound dimmers and oligomers (33). Although the HARP domain involved in the dimerization process is unknown, the positively charged $NH_2$ and COOH termini of HARP, involved in its biological activity (22, 30), might be masked and unable to bind to the HARP receptor. Alternatively, in equal amounts of HARP and peptides, the concentration of the peptides used is higher than that of the whole molecule of HARP. Whether these or similar peptides exist physiologically (e.g. after proteolysis of HARP), is not known at present and is under investigation.

The mechanism of action of HARP is not yet clarified and it is not clear whether its angiogenic effect is due to interactions with cell surface proteoglycans or binding to another cell surface receptor. Several HARP receptors have been reported, the first being N-syndecan (syndecan-3), a transmembrane heparin sulphate proteoglycan which mediates the neurite-promoting signal (14). Another receptor for HARP is the receptor protein tyrosine phosphatase (RPTP)$\beta/\zeta$ (34, 35), which increases tyrosine phosphorylation through ligand-dependent receptor inactivation (35). Concerning HARP mitogenic activity, a high affinity binding site for HARP has been described in NIH 3T3 cells (36) and tyrosine phosphorylation seems to play an important role (37). No specific cell surface macromolecules related to HARP angiogenic activity have been yet identified. Glycosaminoglycans induce HARP dimerisation via covalent bonds, a key event in the activation of the transmembrane signalling receptors of several growth factors (33). It is possible that HARP interaction with endothelial cells may involve both glycosaminoglycans and a transmembrane specific receptor, although it is not clear if and to what extent these interactions are involved in the angiogenic effect induced by HARP. Moreover, it is possible that HARP or its terminal peptides have an indirect effect, potentiating the angiogenic effect of other growth factors or releasing growth factors sequestered in the ECM, such as FGF-2, by inducing stimulation of proteases (22, 38).

In conclusion, our findings emphasise the role of HARP in angiogenesis both *in vivo* and *in vitro*. HARP synthetic peptides representing native molecule residues 1–21 and 121–139 seem to be involved in the angiogenic activity of HARP, suggesting that minimal structures could be sufficient to trigger endothelial cell activation if they are suitably presented to the cell. Interestingly, the effect of HARP and both peptides was qualitatively different among the different angiogenic assays tested.

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# Endothelial Cell Proliferation Induced by HARP: Implication of N or C Terminal Peptides

E. Papadimitriou,* M. Heroult,† J. Courty,† A. Polykratis,‡ C. Stergiou,*‡ and P. Katsoris‡,[1]

*Laboratory of Molecular Pharmacology, Department of Pharmacy, and ‡Division of Genetics, Cell and Developmental Biology, Department of Biology, University of Patras, Patras, GR26504, Greece; and †Laboratory of CRRET CNRS ESA 70-53, University of Paris Val de Marne, Avenue du Général de Gaule, 94010 Créteil, France

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**HARP (Heparin Affin Regulatory Peptide) is a 18-kDa secreted protein displaying high affinity for heparin. It has neurite outgrowth-promoting activity, while there are conflicting results regarding its mitogenic activity. In the present work, we studied the effect of human recombinant HARP expressed in bacterial cells as well as two peptides (HARP residues 1–21 and residues 121–139) on the proliferation of three endothelial cell types derived from human umbilical vein (HUVEC), rat adrenal medulla (RAME), and bovine brain capillaries (BBC) either added as a soluble form in the cell culture medium or coated onto the culture plate. HARP added in a soluble form in the culture medium had no effect on the proliferation of BBC, HUVEC, and RAME cells. However, when immobilized onto the cell culture plate, HARP had a concentration-dependent mitogenic effect on both BBC cells and HUVEC. The peptides presented as soluble factor induced a significant concentration-dependent mitogenic effect on BBC cells but only a small effect on HUVEC and RAME cells. When they were immobilized onto the cell culture plate, the mitogenic effect was much greater. The most responsive cells were BBC that expressed and secreted in the culture medium the higher amounts of HARP.** © 2000 Academic Press

*Key Words:* **HARP; pleiotrophin; HB-GAM; endothelial cells.**

HARP (heparin affin regulatory peptide), also called pleiotrophin or HB-GAM (heparin binding–growth-associated molecule), is a 18-kDa secreted protein with distinct lysine-rich clusters within both the $NH_2$- and COOH-terminal domains (1). HARP has a high affinity for heparin and is localized in the extracellular matrix through interactions with glycosaminoglycans (2–5). It is highly conserved among species (2, 4, 6) and shares 50% homology with midkine and the avian retinoic-induced heparin binding protein (1, 2, 7). These proteins constitute a new heparin-binding growth factor family, structurally distinct from the FGF family (8). HARP is expressed in developing tissues (1, 2, 10, 12) and displays important function in the growth and differentiation processes. In adults, HARP has been found in neuronal tissues, heart, uterus, cartilage and bone (9–12), indicating that it may also have important physiological roles during adulthood. High levels of HARP are detected in speciments of many human tumors, including neuroblastoma, glioblastoma, prostate cancer, lung cancer and Wilms' tumor (13–18). *In vitro,* HARP mRNA is detected in various human cell lines, originally derived from breast cancer, prostate cancer and ovarian carcinoma (19–22).

HARP has an indisputable neurite outgrowth promoting activity (1, 2, 23) while there are conflicting results regarding its mitogenic activity (6, 24, 25), that vary according to the type and the origin of the studied cells (11, 14, 26–28). Only the recombinant polypeptide produced in a mammalian system displayed induction of cellular growth and has been shown to stimulate proliferation of fibroblasts, epithelial and endothelial cells (2, 11, 14). HARP expressed in insect cells or in a bacterial expression system has neurite outgrowth activity but in all studies up to date lacks mitogenic activity, suggesting that the mitogenic and the neurite outgrowth activities are mediated by distinct protein domains of the molecule (24, 29).

In the present study, we investigated the effect of human recombinant HARP expressed in bacterial cells and two peptides that correspond to the amino- and carboxyl-terminals of HARP on the proliferation of three endothelial cell types, when presented to the cells as a soluble factor or as a substrate. We also examined if these cells expressed and secreted HARP into their culture medium and/or deposited it onto their extracellular matrix.

[1] To whom correspondence should be addressed. Fax: 30-61-996300. E-mail: katsopan@upatras.gr.

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## MATERIALS AND METHODS

*Materials.* Cell culture reagents were from Biochrom KG (Seromed, Germany). The affinity purified HARP antibodies were obtained as previously described (30). Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins were obtained from Diagnostics-Pasteur (Marne la Coquette, France). Heparin-agarose was from Sigma. Immobilon P was from Millipore Corporation and the chemiluminescence developing system (ECL) was from Amersham (Pharmacia Biotech). The sequences of the HARP peptides corresponding to the $NH_2$ and the COOH terminus of the protein were $NH_2$-AEAGKKEKPEKKVKKSDCGEW-COOH (HARP residues 1–21) and $NH_2$-AESKKKKKEGKKQEKMLD-COOH (HARP residues 121–139), respectively and were of purity higher than 85% (SYNT:EM, France). All other reagents were of analytical grade and were purchased from Sigma.

*Purification of human recombinant HARP.* *E. coli* BL21 pLys cells transformed with the human HARP-pETHH8 plasmid (kindly provided by P. Bohlen), were cultured at 37 °C in LB media containing 50 μg/ml ampicillin and 50 μg/ml chloramphenicol until a cell density with an absorbance 0.4 at 600 nm was achieved. Expression of the recombinant HARP was induced for 2 h at 37 °C by the addition of 2 mM IPTG. Bacteria were then centrifuged and the pelleted cells were resuspended in 50 mM Tris HCl, pH 7.5 containing 1 mM EDTA, 1 μg/ml of leupeptin, 1 μg/ml pepstatin A and 1 μg/ml aprotinin. After three cycles of freezing/thawing, the lysate was sonicated with six pulses of 20 sec each, centrifuged at 27,000 g for 30 min at 4 °C and the resulting pellet- representing insoluble material and inclusion bodies- was solubilized in 50 mM Tris, pH 8.0 containing 10 mM dithiothreitol, 0.1 M EDTA, 1 M NaCl and 8 M urea. After stirring overnight at 4 °C, insoluble material was removed by centrifugation (27,000 g, 30 min) and the supernatant was dialyzed against 25 mM Hepes, pH 7.4 containing 1 M NaCl at 4 °C for 8 h followed by a second 8 h dialysis against 25 mM Hepes, pH 7.4 at 4 °C. After adjusting the ionic strength to 0.5 M NaCl, the refolded proteins were loaded on a heparin-Sepharose column at flow rate of 30 ml/h. After washing extensively with 50 mM Tris-HCl, pH 7.5 containing 0.5 M NaCl, the bound proteins were eluted with 50 mM Tris-HCl, pH 7.5 containing 2 M NaCl. The eluted proteins were then diluted with 50 mM Tris-HCl, pH 7.5 to an ionic strength of 0.4 M NaCl and loaded on a Mono S column (FPLC system, Amersham Pharmacia Biotech) equilibrated with 50 mM Tris-HCl, pH 7.5. Proteins were eluted from the Mono S column using a linear 0.4 to 1 M NaCl gradient at a flow rate of 0.5 ml/min in 50 mM Tris HCl, pH 7.5. Each collected fraction was quantified for its protein contents with a BCA assay (Pierce) using BSA as standard protein.

*Cell culture.* Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords, cultured as previously described (31) and used at passages 1–5. Rat adrenal medulla microvascular endothelial (RAME) cells were a kind gift of Dr. P. I. Lelkes (University of Wisconsin Medical School, WI), were cultured as previously described and used at passages 19–21 (32). Bovine brain capillary (BBC) endothelial cells were cultured as previously described (26) and used at passages 10–16. Cultures were maintained at 37 °C, 5% $CO_2$ and 100% humidity.

*Western blot analysis of HARP.* The presence of HARP in the cell culture medium and in the extracellular compartments defined as cell surface and extracellular matrix, was investigated as previously described (5). The cells were allowed to grow to confluency in a 56 $cm^2$ tissue culture dish and the medium was changed to 2% fetal calf serum (FCS). Twenty-four hours later, the cells were washed twice with phosphate-buffered saline (PBS) pH 7.4 and subsequently with 20 mM Hepes pH 7.4, containing 2 M NaCl supplemented with 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA and 1 μg/ml aprotinin. The 2 M NaCl washes diluted 1:4, as well as the conditioned medium of the cells were incubated overnight with 100 μl of heparin-agarose at 4 °C with continuous agitation. The heparin-agarose was washed three times with 10 ml of 20 mM Hepes pH 7.4, 0.5 M NaCl and twice with 10 ml of 20 mM Hepes, pH 7.4. Bound proteins were eluted with 50 μl of Laemmli sample buffer under reducing conditions, fractionated on 15% SDS-PAGE and transferred to Immobilon P membranes. Blocking was performed by air-drying the PVDF membranes, according to the manufacturer's instructions. The membranes were incubated with 1 μg/ml affinity purified HARP antibody in Tris-buffer saline (TBS), 0.2% (v/v) Tween-20 for 1 h at room temperature and then with horseradish peroxidase conjugated goat anti-rabbit IgG at a dilution of 1:5.000. Detection of HARP was performed by ECL, according to the manufacturer's instructions.

*Cell proliferation assays.* The growth-promoting activity of HARP and its COOH and $NH_2$ peptides was determined by measuring the number of cells, using the 3-[4,5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide (MTT) assay (33). HUVEC and RAME cells were seeded at $2 \times 10^4$ cells/well and BBC cells at $10^4$ cells/well in 48-well tissue culture plates in the corresponding culture medium supplemented with 2% FCS. HARP or the peptides were added to the medium of the cells at concentrations ranging from 0.001 to 1 μg/ml and the number of cells was measured after 48 h. In another set of experiments, the 48-well tissue culture plates had been previously coated with 0.2 ml of solution of HARP or peptides in carbonate buffer at concentrations ranging from 0.001 to 1 μg/well. Cells were seeded at the same density as above, in the corresponding culture medium supplemented with 2% FCS and their number was determined 48 h later. MTT stock (5 mg/ml in PBS) at a volume equal to 1/10 of the medium was added and plates were incubated at 37 °C for 2 h. The medium was removed, the cells were washed with PBS pH 7.4 and 100 μl acidified isopropanol (0.33 ml HCl in 100 ml isopropanol) were added to all wells and agitated thoroughly to solubilize the dark blue formazan crystals. The solution was transferred to a 96-well plate and immediately read on a microplate reader (Biorad) at a wavelength of 490 nm.

*Statistical analysis.* The significance of variability between the results from various groups was determined by one-way analysis of variance. Each experiment included triplicate wells for each condition tested. All results are expressed as mean ± SEM from at least three independent experiments.

## RESULTS

### *Mitogenic Activity of HARP*

We investigated the effect of human recombinant HARP expressed in bacterial cells as well as two peptides that correspond to the amino- and carboxyl-terminal parts of HARP on the proliferation of three endothelial cell types from three different sources, namely BBC, RAME and HUVEC. Since HARP is also present in the extracellular matrix of cells (2, 5), we also studied the mitogenic effect of HARP or the peptides when they were coated onto the cell culture plates.

HARP added in the cell culture medium of BBC cells had no effect on their proliferation even at a concentration higher than 100 ng/ml. In contrast, both the $NH_2$ and the COOH peptides of HARP used at concentrations between 1 and 1000 ng/ml induced a significant increase (about 80–100% maximum) of cell proliferation (Fig. 1A). Interestingly, a statistically significant effect (up to 70% over the control) was observed when HARP was coated onto the cell culture plate at a concentration higher than 300 ng/well (Fig. 1B). Both HARP peptides, when coated onto the

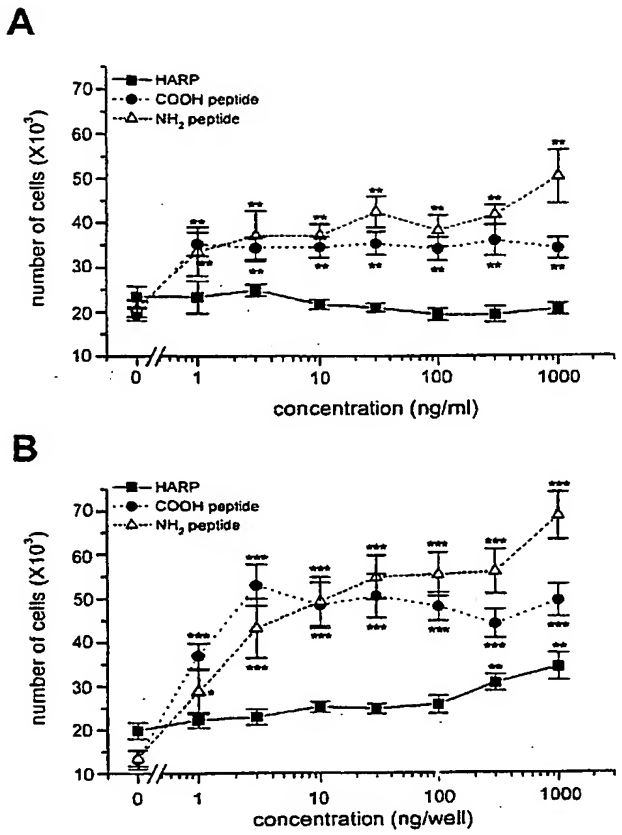


**FIG. 1.** Effect of human recombinant HARP expressed in bacterial cells and two peptides, corresponding to its COOH- and $NH_2$- termini, on the proliferation of BBC cells. HARP or peptides were added at different concentrations to the culture medium of the cells (A) or coated onto the corresponding wells of the cell-culture plates (B). Cells were further incubated for 48 h before measuring their number, as described under Materials and Methods. *, **, and *** denote statistical significance of $P < 0.05$, 0.01, and 0.001, respectively.

cell culture plate, also displayed a concentration-dependent mitogenic effect on BBC cells, which was much higher than the corresponding effect when the peptides were added as soluble molecule into the cell culture medium (Fig. 1). A highly significant effect was observed at a concentration lower than 3 ng/well for both peptides and the maximum increase of the number of cells was about 300% for the COOH peptide and 400% for the $NH_2$ peptide (Fig. 1B).

HARP, as well as both the $NH_2$ and the COOH peptides added in the cell culture medium of RAME cells, had no effect on their proliferation (Fig. 2A). In contrast, when the $NH_2$ or the COOH peptides were immobilized onto the cell culture plate, a concentration-dependent mitogenic effect was observed. The maximum increase, observed at 3 ng/well, was about 90% over the control for both peptides.

No effect on cellular proliferation was observed when HARP or the COOH peptide were presented as a soluble molecule in the cell culture medium of HUVEC. As shown in Fig. 3A, the $NH_2$ peptide had a small (about 30% over the control) and marginally statistically significant mitogenic effect on HUVEC. In contrast, when immobilized onto the cell culture plate, HARP had a concentration-dependent and statistically significant mitogenic effect on HUVEC (maximum 190% at a concentration of 10 ng/well). Both HARP peptides immobilized onto the cell culture plate displayed a concentration-dependent mitogenic effect (Fig. 3B). The maximum increase was observed at a concentration of 30 ng/well for both peptides and the stimulation was about 100% over the control.

### *Presence of HARP in the Extracellular Compartments and in the Culture Medium of Endothelial Cells*

HARP is secreted by cells into the culture medium as well as sequestered in their extracellular compart-

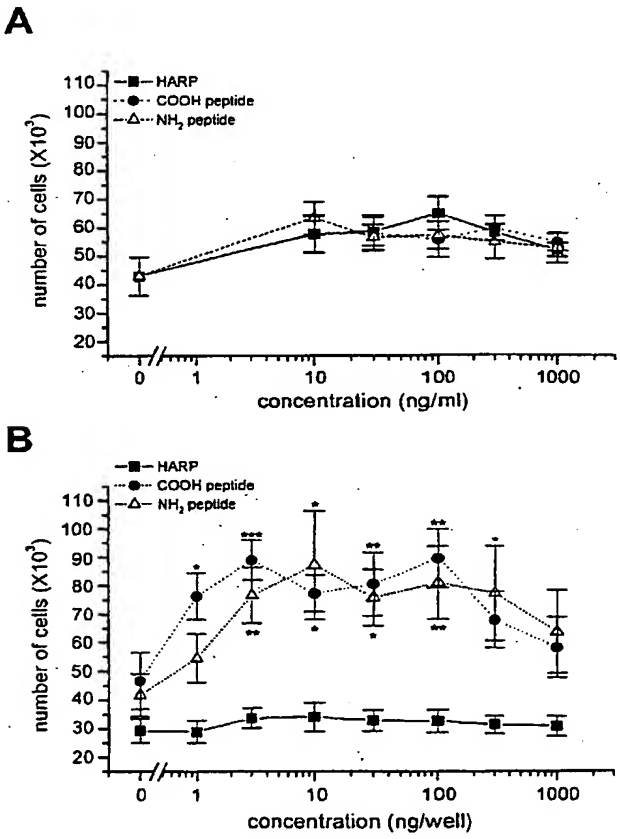


**FIG. 2.** Effect of human recombinant HARP expressed in bacterial cells and two peptides, corresponding to its COOH- and $NH_2$- termini, on the proliferation of RAME cells. HARP or peptides were added at different concentrations to the culture medium of the cells (A) or coated onto the corresponding wells of the cell-culture plates (B). Cells were further incubated for 48 h before measuring their number, as described under Materials and Methods. *, **, and *** denote statistical significance of $P < 0.05$, 0.01, and 0.001, respectively.

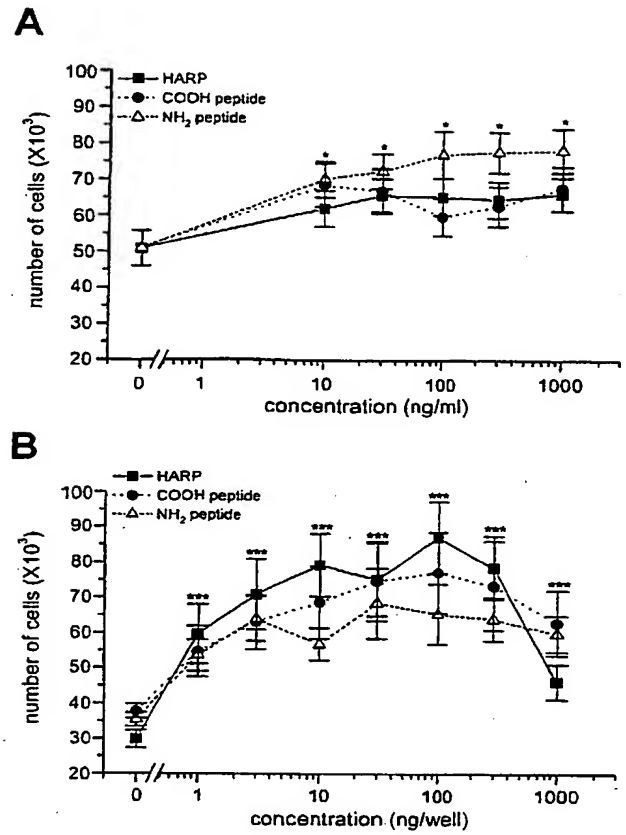


FIG. 3. Effect of human recombinant HARP expressed in bacterial cells and two peptides, corresponding to its COOH- and $NH_2$-termini, on the proliferation of HUVEC cells. HARP or peptides were added at different concentrations to the culture medium of the cells (A) or coated onto the corresponding wells of the cell-culture plates (B). Cells were further incubated for 48 h before measuring their number, as described under Materials and Methods. * and *** denote statistical significance of $P < 0.05$ and 0.001, respectively.

ments including matrix and cell surface (4, 5). The difference in the response of the endothelial cell types to HARP and its related peptides led us to determine whether these cells secreted and/or deposited this molecule and to what extend. Western blot analysis showed that HARP was secreted in the culture medium of BBC cells (Fig. 4, lane 2), while it was not detectable in the culture medium of RAME cells (Fig. 4, lane 4) and HUVEC (Fig. 4, lane 6). HARP was also detected in the 2 M NaCl washes of the surface of BBC (Fig. 4, lane 1) and RAME cells (Fig. 4, lane 3), which correspond to the amounts of HARP bound to the extracellular compartments. No signal was detected in the extracellular compartments of HUVEC (Fig. 4, lane 5). BBC produced much higher amounts of HARP than RAME cells. Comparing the profiles of HARP secreted in the culture medium and deposited on the matrix of the cells, it was obvious that BBC secreted higher amounts of HARP than RAME cells, which only secreted a smaller than 18 kDa form recognized by the anti-HARP antibody. This form was also detected in the cell fraction of both BBC and RAME cells. Interestingly, high molecular HARP immunoreactivity (27 kDa, 36 kDa and 50 kDa) was detected in the culture medium of BBC and the extracellular compartment of both BBC and RAME cells.

## DISCUSSION

In the present study we investigated the effect of human recombinant HARP expressed in bacterial cells and two synthetic peptides (HARP residues 1–21 and 121–139) on the proliferation of three endothelial cell types. Whatever the endothelial cell type, HARP had no significant effect on endothelial cell proliferation when it was added in the cell culture medium. These data agree to the previously published studies (2, 12, 25, 30) according to which only HARP produced in eucaryotic expression systems has mitogenic activity (2, 12), while HARP produced in prokaryotic expression systems lacks or has limited mitogenic effect (25, 30), due possibly to incorrect folding or incomplete processing of the recombinant polypeptide. Interestingly, however, when HARP expressed in bacterial cells was immobilized onto the cell culture plate, a dose-dependent mitogenic effect was observed in two of the three endothelial cell types studied. HARP binds with high affinity to heparin and thus to glycosamino-

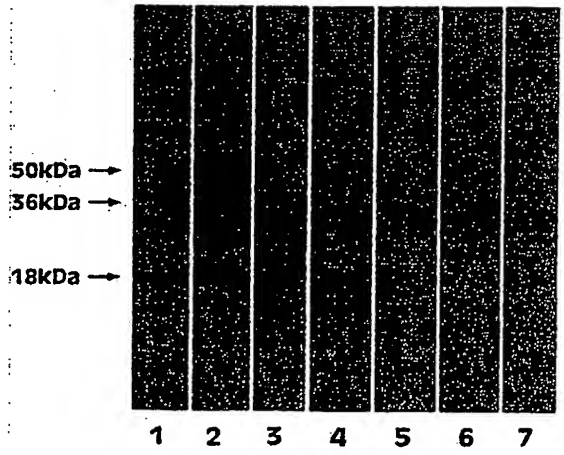


FIG. 4. Production and secretion of HARP by endothelial cells. (1) BBC cells and extracellular matrix bound material. (2) Conditioned medium of BBC cells. (3) RAME cells and extracellular matrix-bound material. (4) Conditioned medium of RAME cells. (5) HUVEC and extracellular matrix bound material. (6) Conditioned medium of HUVEC. (7) DMEM supplemented with 2% FCS. Conditioned media and the NaCl washes of the cells were collected as described under Materials and Methods and were incubated with 100 $\mu$l of a 10% suspension of heparin-agarose. Bound protein was eluted with Laemmli sample buffer and analyzed by SDS-PAGE followed by Western blot analysis, using an affinity-purified anti-HARP antibody.

glycans, including heparan sulfate (5, 35–37) and dermatan sulfate (5) derived from extracellular matrix, functioning possibly as a mitogenic molecule trapped in the extracellular space. A similar stimulating effect of HARP has been reported also for its *in vitro* neurite outgrowth activity. In that work, HARP was more effective when presented to chick embryo cerebral cortical derived neurons as a substrate than as a soluble factor (7). Endothelial cells exhibit polarized secretion of extracellular matrix molecules (32, 37) and localized matrix assembly sites (37). It is thus possible that the molecules on the endothelial cell surface involved in the binding and the transmission of HARP mitogenic signal are localized preferably on the basolateral side rather than the apical surface of the cells. A recent publication suggested that the binding of HARP to heparin was much tighter when HARP was immobilized on a surface (36). Thus, an alternative explanation to our results is that when HARP is immobilized onto the cell culture plate, it binds tighter to the cell surface glycosaminoglycans and thus exerts its mitogenic effect better than when it is presented in soluble form in the cell culture medium.

From the present study, it is not clear whether the mitogenic effect of HARP is due to interactions with cell surface proteoglycans or binding to another cell surface receptor. HARP receptors have been reported only for its neurite outgrowth activity. N-syndecan (syndecan-3), which mediates the neurite-promoting signal, is a transmembrane heparin sulfate proteoglycan that also binds other heparin-binding growth factors, like basic fibroblast growth factor (35). Another putative receptor for HARP is 6B4 proteoglycan/phosphacan, a truncated form of a receptor protein tyrosine phosphatase (RPTP)$\beta/\zeta$ (39). No specific cell surface macromolecules related to HARP mitogenic activity have been yet identified. Several studies have reported the existence of a high affinity binding site for HARP in several cell types including NIH 3T3 cells (24, 40) and bovine epithelial lens cells (41), where HARP action requires the activation of tyrosine kinase of the mitogen-activated protein kinase and PI3-kinase pathways (41). Heparitinase treatment of cells reduced HARP-induced cellular proliferation (5, 24) and glycosaminoglycans induced HARP dimerization, a key event in the activation of the transmembrane signaling receptors of several growth factors (34). It is possible that HARP interaction with cells may involve both glycosaminoglycans and a transmembrane specific receptor although it is not clear to what extent these interactions are involved in the mitogenic effect induced by HARP. Moreover, the diverse response of the different studied cells indicates that such interactions can vary among the cells, fact that renders the phenomenon even more complicated.

Interestingly, both peptides (HARP residues 1–21 and 121–139), had a concentration-dependent mitogenic effect, which was much greater when the peptides were immobilized onto the cell culture plate. It seems that the two lysine-rich terminal domains of HARP are implicated in multiple biological actions, since they stimulate endothelial cell proliferation (this paper) and are required for successful transformation of NIH 3T3 cells by HARP residues 41–64 (42). Both terminal regions account for the ability of HARP to bind tightly to heparin and to extracellular matrix (2, 4) and either of them was required for transformation of NIH 3T3 cells by HARP residues 41–64 (42), indicating that these domains have a similar functional role. This is also the case in our results, where the two peptides always had similar effect on endothelial cell proliferation. It has been hypothesized that the positively charged $NH_2$ and COOH-termini of HARP interact with the receptor or other molecules on the cell surface and facilitate HARP binding to a high affinity receptor (42). The existence of a high affinity HARP receptor, similar to the receptors of other heparin binding growth factors, has been strengthened by the finding that HARP dimerizes in the presence of glycosaminoglycans (34). In the present study, both HARP peptides had by themselves a significant effect on endothelial cell proliferation, which in the case of BBC and RAME cells was even higher than the effect of HARP itself. These results suggest that the peptides themselves bind to and activate the transmission of signals inside the cells. When both peptides were introduced simultaneously to the cells, the mitogenic effect was similar to that induced by each peptide alone, even at concentrations that did not induce maximal effect of each (data not shown). It seems that both peptides interact with the same molecule on the cell surface to induce endothelial cell proliferation with similar mechanism. Whether these or similar peptides exist physiologically, e.g. after proteolysis of HARP under certain circumstances, is not known at present and is under investigation.

It is not clear from our data whether the mitogenic effect of HARP or its peptides is due to an indirect mechanism involving displacement of other matrix-bound growth factors or to the interactions with specific cell surface receptor(s). Several studies have characterized the interaction of highly basic peptide including the exon 6-encoded sequence of VEGF (43) and the exon 6 of PDGF A chain (44) with cell surface glycosaminoglycans. These synthetic peptides also prevented the binding and the mitogenic activity of exogenously added growth factors like FGF-2 or PDGF-AA (45). In addition, it has been shown that the exon 6-encoded sequence of VEGF released iodinated FGF-2 bound to endothelial cells surface and was able to induce an angiogenic response in a corneal pocket assay, which was totally blocked by addition of anti-FGF-2 antibodies (43). These observations demonstrated that basic peptides such as axon 6-encoded sequence of

VEGF exert its mitogenic activity through FGF-2 signaling pathways. Due to their highly basic sequence, the HARP residues 1–21 and HARP residues 121–139 might be acting through an indirect mechanism involving de-sequestration of other matrix-bound growth factors.

The cells most responsive to HARP were BBC, which were those that expressed much higher amounts of HARP than the other studied cells. These cells derive from brain, where HARP has a functional role (11) and it is also possible that it is important for the growth of these cells as well. Interestingly, we have transfected BBC cells with anti-sense HARP mRNA but none of the transfections was successful because of cell death (unpublished observation). Control transfection with HARP sense mRNA continuously gave alive transfected cells. Another explanation could be that different cell surface molecules interact with HARP among the different cells, as discussed above.

In conclusion, human recombinant HARP expressed in bacterial cells had no mitogenic effect when presented as a soluble factor, while it is mitogenic when presented to endothelial cells as a substrate. HARP residues 1–21 and 121–139 seem to be involved in the mitogenic activity of HARP, suggesting that minimal structures could be sufficient to trigger endothelial cell proliferation if they are suitably presented to the cell. Interestingly, the effect of HARP and both peptides was quantitatively different among the different endothelial cells tested.

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